NEMATOCYSTS OF *MICROSTOMA*.

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For a long time the nematocysts of *Microstoma* were considered to be structures that the worm elaborated as did *Hydra*. Martin ('08) published an account of the manner in which *Hydra* is attacked by *Microstoma* and indicated his conviction that the namatocysts of *Microstoma* were derived from the Hydras that had been eaten. Kepner (11) published an account of the histological details involved in the process of transporting the nematocysts of *Hydra* from the enteron to the epidermis of *Microstoma*. In this account the inference was made that this complicated series of phenomena must mean that *Microstoma* handled these nematocysts in this manner in order to use them. This inference was early challenged by Glaser (11). A tentative reply was given Glaser's note in *Science*, Volume 34, page 213, 1911.

The details of the processes involved when *Microstoma* deals with the nematocysts are so variable and so intricate that it was felt that these processes could mean only one of two things, namely, either this was a method of eliminating indigestible foreign bodies or a method of securing weapons of defense or perhaps offense.

From time to time since 1911 efforts have been made to determine which of the two meanings was behind this conduct of *Microstoma*. This paper represents the results of our efforts that have thus extended over a decade and have been taken up each year of this decade to a certain extent.

MATERIAL AND METHODS.

Microstoma caudatum is found rather abundantly in the early spring months in the submerged detritus along the banks of fresh water ponds about the University of Virginia. Sometimes Hydras are found living with it in great numbers; at other times

there are few Hydras. When many Hydras are present it is difficult to find Microstomas that lack nematocysts. When there are few Hydras living with *Microstoma* in nature, specimens that lack nematocysts are readily obtained.

It has been found that by placing *Microstoma* in watch-glasses containing fresh water and some fragments of water soaked, dead leaves they can be kept indefinitely; provided the air be kept free of coal gas, illuminating gases, and formaldehyde vapor.

Nematocysts Passing from Endoderm into Mesoderm.

While this paper is not dealing with the methods by which *Microstoma* handles nematocysts, it is of interest to record that Kepner and Whitlock in 1917 observed a nematocyst being carried down through the body of an endodermal cell and delivered through the basement membrane into the mesenchyme. This was a very slow process. The movement of the nematocyst could be appreciated only when its position was recorded and then five minutes later its position again observed. Its rate was not accelerated as it passed through the basement membrane into the mesoderm. The most remarkable feature of this was that the obtuse end was directed towards the path along which the nematocyst was being transported.

Nematocysts Uniformly Distributed.

Attention might be called to the additional fact that when these nematocysts are taken up by the mesenchymal cnidophages they are distributed uniformly at the epidermis by these attending cells. So that immediately after a *Microstoma* has acquired a supply of nematocysts at its surface, these nematocysts are uniformly distributed. It does not appear, however, that if the anterior end, or any other region, lose its quota of nematocysts that some will be taken from other regions of the body to take the place of those lost. In this manner specimens sometimes come to have a uniform distribution of nematocysts except for some region (e. g. "head") that is free from them. Despite this fact, the subtle manner in which the cnidophages coöperate to bring about a uniform distribution of the foreign nemato-

cysts is remarkable and lends weight to the inference that the nematocysts are being collected by the *Microstoma* to be used.

Nematocysts Retained Indefinitely.

Again if the nematocysts of *Hydra* are carried out to the surface of *Microstoma* by way of eliminating them as indigestible foreign bodies, the question arises as to why they are held so long at the surface. Within twelve hours after a *Hydra* has been ingested by *Microstoma,* the nematocysts are distributed over the rhabdocoele's body. The process of getting these objects to the surface of the flatworm is, therefore, a matter of about twelve hours. When, however, they arrive at the surface they are held there indefinitely. Even when animals are kept under adverse conditions in the laboratory and they show more and more conspicuous decline, the nematocysts are yet retained. Specimens have been seen growing weak and beginning to rupture or break up and yet retain the nematocysts over the epidermis that remained intact. This retention of the nematocysts, likewise, suggests that they have been collected for use.

An Expensive Method.

Again this is an expensive method of dealing with objects that could be thrown out at the mouth as are other indigestible materials. For in this process much energy must be spent by the endodermal cells and cnidophages in handling the nematocysts; and, further, when the nematocysts are discharged they carry with them their attending cnidophages. The cnidophages, therefore, in attending the nematocysts act against their own welfare in that in the end they lose their own lives.

Microstoma Incurs a Danger.

Even the *Microstoma* as a whole feeds upon *Hydra* at a risk. For sometimes *Hydra* turns upon *Microstoma* and eats it. *Hydra,* however, seems to have difficulty in ingesting *Microstoma* as the following observation of January 18, 1917, shows. The *Microstoma* at once played along the side of *Hydra viridis* that had been placed with it. The worm passed to and fro along the surface of the polyp's body. Twice it came to rest amidst

the bases of the tentacles. The third time it came to lie over the mouth of the *Hydra,* the latter began an effort to ingest the *Microstoma* (Fig. 1). First the *Hydra's* peristome opened up against the ventral side. This region of the peristome did not fix the *Hydra* to the *Microstoma,* for the mouth of *Hydra* glided posteriory along the ventral side of *Microstoma* and around its posterior end as indicated by the arrow in Fig. 2. When the widely expanded peristome of *Hydra* had come to be applied over a great part of the dorsal and lateral surfaces of the *Microstoma,* the *Hydra* pressed the latter between its peristomal bell and its body and appeared to have the worm in a very serious position (Fig. 3). But from this embrace the *Microstoma* soon glided. As it escaped it showed a ruptured region of its body. This wound healed in a little while and then *Microstoma* went back and played along the surfaces of *Hydra's* body. The next time it came into the tentacular zone of *Hydra,* the polyp succeeded in grasping the *Microstoma* head-on and forthwith ingested it (Fig. 4). Since, therefore, *Microstoma* incurs a greater danger in seeking out *Hydra* than it does when it feeds upon small annelids, crustacea and other small animals and plants and since, further, it does not appear to seek *Hydra* primarily for food as will be shown later, the inference is strong that it is seeking *Hydra* for some unique end.

Microstoma Able to Draw Nematocysts from its Body when Wounded by *Hydra.*

Moreover, *Microstoma* seems to have developed an adaptive secretion by its epidermal glands that may be taken to be either rhabditic glands or the homologues of such glands which are found in other rhabdocoeles. The following observations indicate the adaptive functioning of these glands. February 15, 1917, a *Microstoma* was placed with a small *Hydra* fusca. The *Microstoma* began playing about the five tentacles. Immediately the *Hydra* firmly grasped it between its tentacles (Fig. 5). The *Microstoma* escaped from the *Hydra's* embrace. As it swam clear of the polyp, two mucous masses were to be seen, one at the posterior end and one on the right side. (Fig. 6). The lateral mass grew and as it grew drew the two enclosed nemato-

cysts out from their anchorage within the worm's body and then glided along the right side of the body posteriorly to where it fused with the posterior mass, which had three nematocysts enclosed within it. This compound mass of mucus, now containing five freed nematocysts, was sloughed off from the *Microstoma's* body and left behind. After that the *Microstoma* seemed to be enveloped in a transparent mucus sheath to which small dead objects adhered and were dragged about with the *Microstoma* until they became entangled with the tentacles of the *Hydra*. These tentacles stripped the foreign bodies from the *Microstoma's* adhesive surface. Another observation of this sort was made January 29, 1917. A *Microstoma* was placed with a large budding *Hydra fusca*. It at once attacked the *Hydra*. The *Hydra* discharged a nematocyst into the anterior end of *Microstoma*. The *Microstoma* contracted so vigorously as to break apart the two nearly formed zooids that had been developing through fission. About the deeply anchored nematocyst the anterior zooid now secreted a local mass of mucus. This mass of mucus grew until it formed a column whose length was more than two-thirds that of the zooid. Next the posterior zooid encountered the bud of the *Hydra*. As it passed the *Hydra*, the latter stung it in its posterior end along the left side. About this nematocyst a local secretion of mucus appeared and as the mass of mucus grew in length the long stinging thread of the nematocyst was dragged from the *Microstoma's* body as had a mucous column dragged the nematocyst from the body of the anterior zooid. Eventually the mucous masses were discarded and the two zooids appeared to be no worse for their experience.

All the above shows an intimate relation existing between *Microstoma* and *Hydra* which is peculiar and must have some meaning while much of the above indicates that the meaning of this conduct centers about the nematocysts of *Hydra*.

Microstoma with Few Nematocysts Reacts to *Hydra* Readily.

Moreover, a *Microstoma* that has few or no nematocysts behaves differently towards *Hydra* than does one that has a com-

plete quota of nematocysts at its surface. On December 8, 1916, a *Microstoma,* that contained seven or eight nematocysts and so greatly filled with food that the pharynx of its posterior zooid was everted, was placed with a *Hydra.* Within 12 minutes the *Microstoma* had egested some of its food and had torn off a part of the *Hydra's* oral end. Two days later a second well-fed specimen, that contained but two nematocysts, was placed with a *Hydra.* This specimen ate the *Hydra* within twenty minutes. Table I., shows that of 42 specimens containing none or few nematocysts, 6 (within two minutes after being placed with Hydras) set to work trying to feed upon the polyps, but five of them got severely wounded while the sixth was eaten by the *Hydra.* Two others were eaten by the *Hydra* within twenty-four hours. Of the remaining 34 specimens, 9 had eaten Hydras in periods ranging from 2 minutes to 20 minutes; while 20 accepted Hydras in periods ranging from 24 hours to 1 hour; and 3 remained with *Hydra* either 5 or 2 days before they accepted *Hydra.* Two specimens remained with *Hydra* 24 hours and a third 4 days when they were lost or had died without having accepted *Hydra.* The conspicuous feature of Table I. is that in many cases *Hydra* was accepted within a period of minutes, many others within a period of hours, while only a few were accepted within a period of days.

Microstoma with Many Nematocysts Reacts to *Hydra* Tardily.

Table II. makes a sharp contrast with Table I. in this respect. For this table shows the reactions of 18 Microstomas, that had either many or a full quota of nematocysts. All, except specimen 15, were kept from food for a day or more and yet only one of them, specimen 7, reacted to *Hydra* within 24 hours and this one contained only about one-third of a full quota of nematocysts. Specimen 8 remained with *Hydra* 24 hours when it was lost, without having accepted *Hydra.* Except for these two specimens, all the others reacted to *Hydra* not within periods of hours or minutes but of days and that despite the fact that they had no food for at least a day. Specimens 17 and 18 were kept away from food in each case four days. They lived four

TABLE I.

NEMATOCYSTS OF *Microstoma* NONE OR FEW.

Specimen.	Number of Nematocysts.	Time with *Hydra*.	*Hydra* Eaten.	*Hydra* Not Eaten.	*Microstoma* Eaten.	*Microstoma* Stung.
11–21–16.....	None	24 hrs.	+			
12– 6–16.....	7 or 8	12 min.	+ though the specimen had much food.			
12–10–16.....	2	20 min.	+ though well fed.		–	–
12–11–16.....	6	3 min.	+	–	–	–
1–10–17.....	None	24 hrs.	+	–	–	–
1–18–17.....	2	2 min.	–	–	+	–
1–20–17.....	6–10	2 days	+	–	–	–
1–23–17.....	8	5 days	+	–	–	–
1–24–17.....	5	2 min.	–	–	–	+
1–26–17.....	1 or 2	2 min.	–	–	–	+
2–12–17.....	1	2 min.	–	–	–	+
4–19–17.....	10	2 min.	–	–	–	+[1]
7–23–17.....	Few	2 min.	–	+	–	+
7–24–17*a*.....	6	10 min.	+	–	–	–
7–24–17*b*.....	None	10 min.	+	–	–	–
8–18–17*b*.....	None	5 min.	+	–	–	–
9–18–17*d*.....	1	24 hrs.	+	–	–	–
9–18–17*e*.....	Few	24 hrs.	–	+	+	–
9–18–17*f*.....	2	24 hrs.	+	–	–	–
9–18–17*g*.....	7	24 hrs.	+	–	–	–
9–18–17*h*.....	Few	5 min.	+	–	–	–
9–19–17*a*.....	Few	24 hrs.	+	–	–	–
9–19–17*b*.....	1	24 hrs.	+	–	–	–
9–19–17*c*.....	None	24 hrs.	+	–	–	–
9–19–17*d*.....	5	24 hrs.	+	–	–	–
9–19–17*e*.....	5	24 hrs.	+	–	–	–
9–19–17*f*.....	None	1 hr.	+	–	–	–
9–19–17*g*.....	7	1 hr.	+	–	–	–
9–19–17*h*.....	None	3 min.	+	–	–	–
10– 5–17*a*.....	None	6 hrs.	+	–	–	–
10–17–17*a*.....	1	24 hrs.	+	–	–	–
10–17–17*c*.....	6–8	24 hrs.	+	–	–	–
10–17–17*e*.....	None	24 hrs.	–	+	–	–
10–17–17*f*.....	None	24 hrs.	+	–	–	–
10–17–17*h*.....	8–10	24 hrs.	+	–	–	–
10–17–17*j*.....	None	24 hrs.	–	+	–	–
10–17–17*l*.....	Few	24 hrs.	–	+	–	–
10–17–17*m*.....	Few	24 hrs.		–	+	–
9–18–17*e*.....	Few	2 days	+	–	–	–
5– 3–23.....	Few	4 days	–	+	–	–
5– 3–23.....	10	24 hrs.	+	–	–	–
5– 8–23.....	None	24 hrs.	+	–	–	–

days with *Hydra* before they died without accepting any part of *Hydra*. Specimen 16 is the most significant one of the table. It was learned by observation that *Microstoma* could live in a small vessel of spring water for about nine days without food. Many died in less time under these conditions but none passed

[1] Rather by gastric juices than by nematocysts.

TABLE II.

NEMATOCYSTS OF *Microstoma* MANY.

Specimen.	Loaded with Nemato-cysts.	With-out Food.	With *Hydra*.	*Hydra* Eaten.	*Hydra* Not Eaten.	Nematocysts Thrown from Enteron of *Microstoma*.
(1) 9–18–17*B*	1/2	1 day	2 days	+	−	−
(2) 9–18–17*D*	1/2	"	"	−	+	Sick; see notes.
(3) 9–18–17*F*	4/5	"	"	+	−	−
(4) 9–18–17*G*	1/2	"	"	+	−	−
(5) 9–19–17*B*	1/3	"	"	+	−	−
(6) 9–19–17*C*	1/2	"	3 days	+	−	−
(7) 9–19–17*D*	1/3	"	1 day	+	−	−
(8) 9–19–17*D*	Loaded	"	"	−	+	−
(9) 9–19–17*E*	2/3	"	3 days	+	−	−
(10) 9–19–17*F*	1/4	"	"	−	+	− Sick; see notes.
(11) 9–19–17*G*	1/4	"	"	+	−	−
(12) 9–19–17*H*	Loaded	"	"	−	+	
(13) 9–19–17*H*	4/5	"	2 days	+	−	+
(14) 9–19–17*A*	1/2	"	"	+	−	−
(15) 9–19–17*A*	Loaded	No[1]	"	+	−	+
(16) 11–21–16	Loaded	9 days	"	−	+	Piece of tadpole liver
(17) 5– 6–23	1/2	4 days	4 days	−	+	accepted immediately
(18) 5–10–23	1/5	"	"	−	+	after the *Microstoma* was taken from the *Hydra* of specimen (16).

the twelfth day. With this fact in mind a "loaded" specimen was kept away from food for nine days. It was placed with *Hydra* at the end of the ninth day and remained with it for two more days without accepting *Hydra*. This was not due to the *Microstoma's* condition having been so greatly lowered that it could not accept food. For when it was taken from *Hydra* and given a piece of tadpole liver, the *Microstoma* immediately accepted it. The facts tabulated in these two tables indicate that a *Microstoma* with few or no nematocysts attacks a *Hydra* much more readily than does one with many or a full quota.

Microstoma MAY EGEST CELLS OF *Hydra* AND RETAIN NEMATOCYSTS.

Another contrast may be drawn between the conduct of a *Microstoma* that has few or no nematocysts and that of one that has a full quota of nematocysts. If a *Microstoma* that contains a *Hydra,* so long ingested that the polyp has been reduced to a pulp, be placed under slight pressure it will discharge

[1] Not starved since it had eaten *Hydia* as specimen 14.

the green protoplasmic mass of the *Hydra's* body and retain the nematocysts within its enteron. This does not appear to be due to the fact that the pressure has held fast the mass of nematocysts; for, during the process of egesting the other *Hydra* material, the nematocysts are being thrust to and fro within the enteron. Under such condition a *Microstoma,* therefore, rejects the food and retains the nematocysts.

Microstoma May Egest Nematocysts and Retain Cells of *Hydra.*

The senior author saw just the reverse of this. A *Microstoma* was loaded with nematocysts by being fed a *Hydra.* It was then starved until it would accept *Hydra.* While the *Microstoma* was kept under observation the *Hydra* was digested. When alimentation had been completed the indigestible nematocysts were thrown out of the mouth and rejected. When Dr. W. H. Taliaferro, now of Johns Hopkins University, was told this he expressed skepticism; his challenge was accepted by the senior author to have this demonstrated. From 10.58 A.M. to 11.14 A.M., September 19, 1917, Dr. Taliaferro kept the "loaded" *Microstoma* under observation. During this time he could see the nematocysts of a recently ingested *Hydra* within the enteron. At 11.14 A. M., he saw the nematocysts being discharged from the mouth of the "loaded" *Microstoma.*

From these observations it appears, therefore, that *Hydra* is eaten by *Microstoma* not primarily as a food but for its nematocysts. Thus, it is further suggested that the handling of the nematocysts by *Microstoma* is done in order that the rhabdocoele may use these "stinging threads."

Discharge of Nematocysts by Microstoma a Double Process.

When one teases a *Microstoma,* that is armed with *Hydra's* nematocyst, the nematocyst in the immediate neighborhood of the stimulus oscillates to and fro at right angles to the surface of the body. If the teasing is maintained long enough, the nematocyst will be discharged at the object with which the *Microstoma*

was being touched. An observer cannot watch this double reaction on the part of the *Microstoma* when being stroked with a needle point without feeling that the first phase of reaction, viz., the oscillation of the nematocyst, is a threat and that the second phase of the reaction, viz., the discharge of the nematocyst, was an effort to use the nematocyst against the annoying object.

Microstoma Uses Its Nematocysts.

Finally we have been able to see *Microstoma* actually using the nematocysts that it had appropriated from *Hydra.*

October 13, 1917, the senior author placed some Stenostomas in a hanging drop with a *Microstoma* that had nematocysts. One of the Stenostomas was at once stung along its side. The wound caused the Stenostoma's body to rupture. After the *Microstoma* had thus wounded the victim, it at once swallowed it. Mr. Conway Zirkle saw this same *Microstoma* strike another *Stenostoma* in such fashion as to cause it to bend its body near the middle at right angles and to remain quiet in this contour until the *Microstoma* fell to ingesting it. In neither of these observations were the actual nematocysts seen entering or having entered the *Stenostoma.*

But on September 9, 1917, the senior author placed a large *Microstoma,* containing nematocysts, in a hanging drop of water with three very large dividing Stenostomas. The head of one of these Stenostomas came in contact with the right side of the *Microstoma's* head. Forthwith the *Stenostoma* contracted violently. From a wound at the tip of the body the mesenchyme oozed. As this material ran out of the body, it dragged with it two nematocysts that had their filaments and barbs ejected. The *Stenostoma's* body also developed a small blister to the left of the ruptured region of the epidermis. The *Microstoma* left the *Stenostoma,* which no longer moved from place to place, and later came back and attempted to ingest the *Stenostoma* as it lay struggling as though suffering from its wound.

September 19, 1917, the senior author placed a *Microstoma,* that had nematocysts, in a hanging drop of water with a single dividing *Stenostoma.* Eight times these two animals collided.

Sometimes these collisions caused the one animal to glide down along the side of the other; at other times the collisions would be head on. In each case the collisions were so evident as to cause a shunting of one or the other specimen. At 11.30 A.M. while *Microstoma* lay quiet, *Stenostoma* made a contact with *Microstoma* and stroked the entire left side of the *Microstoma* with its right ciliated pit. When *Stenostoma's* head had come to be in contact with the posterior end of *Microstoma,* the latter discharged two nematocysts into the region of *Stenostoma's* right ciliated pit. At once a rupture took place in the *Stenostoma's* epidermis and from this wound granular material oozed. Despite this breaking of the *Stenostoma's* body in the region in which it had been stung, the *Stenostoma* was anchored to *Microstoma* by the nematocysts, the poison sacs of which were yet held within the *Microstoma.* The two animals were anchored thus long enough for me to call Dr. I. F. Lewis to my side and make a demonstration of the situation to him. Soon after this demonstration was made, the two poison sacs left the *Microstoma's* body and the latter swam away. The *Stenostoma,* which up to the time it was stung had been incessantly active, lay quite inactive for ten minutes with two nematocysts hanging from the wound on the right side of its anterior end. After that it gradually recovered itself.

It has thus been demonstrated that *Microstoma* uses the nematocysts which it takes from the *Hydra.*

Summary.

1. *Microstoma* manipulates the nematocysts of *Hydra* either (*a*) as a means of eliminating indigestible parts of its food, or (*b*) a means of defending itself.

2. The fact that the nematocysts are distributed uniformly over the surface of *Microstoma* within 12 hours after a *Hydra* has been ingested, suggests that this is not a process of elimination, (*a*), but is done in order that the nematocysts may be used, (*b*).

3. The fact that the nematocysts are retained indefinitely suggests that they have been taken up and retained for use.

4. The fact that this method of handling the nematocysts demands considerable work on the part of the endodermal cells and, in each case of a discharged nematocyst, the death of a cnidophage or mesodermal cell further suggests that it is all done with reference to use.

5. *Microstoma* incurs danger in attacking *Hydra;* for it is frequently stung and sometimes killed by the polyp. This too suggests that the *Microstoma* attacks *Hydra* for some peculiar end.

6. The *Microstoma* is able to draw nematocysts with which *Hydra* has stung it from its body, without suffering a rupture as does *Stenostoma.*

7. *Microstoma* with a few nematocysts attacks *Hydra* within a little time.

8. *Microstoma* having many nematocysts does not readily attack *Hydra.* The contrast between 7 and 8 suggests that *Microstoma* attacks *Hydra* not for food; but for its nematocysts.

9. The alimentary canal of a recently fed *Microstoma,* that has few nematocysts, may egest the "flesh" of *Hydra* and retain its nematocysts.

10. The alimentary canal of a *Microstoma* that has many nematocysts and is hungry, may egest the nematocysts of *Hydra* and retain the "flesh" of the polyp. The contrast between 9 and 10 suggests that *Microstoma* seeks primarily the nematocysts of *Hydra.*

11. The discharge of the nematocysts is a double process, involving (1) an oscillation to and fro of the nematocyst within its cnidophage, and (2) the actual discharge. The first phase may be carried on without being followed by the second phase. Neither of these has ever been seen except when some active body, like the experimenter's needle or an animal has brushed along the surface of *Microstoma.* This, too, suggests that the nematocysts are for use.

12. Finally *Microstoma* actually stings and paralyses other animals with the nematocysts it has appropriated from Hydras that it has eaten.

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EXPLANATION OF FIGURES.

Plate I.

Fig. 1. A *Microstoma* slowly moving amid the bases of the tentacles of *Hydra*. × 50.

Fig. 2. In response to the presence of *Microstoma* the *Hydra* has spread its expanding peristome along ventral surface of *Microstoma*. × 50.

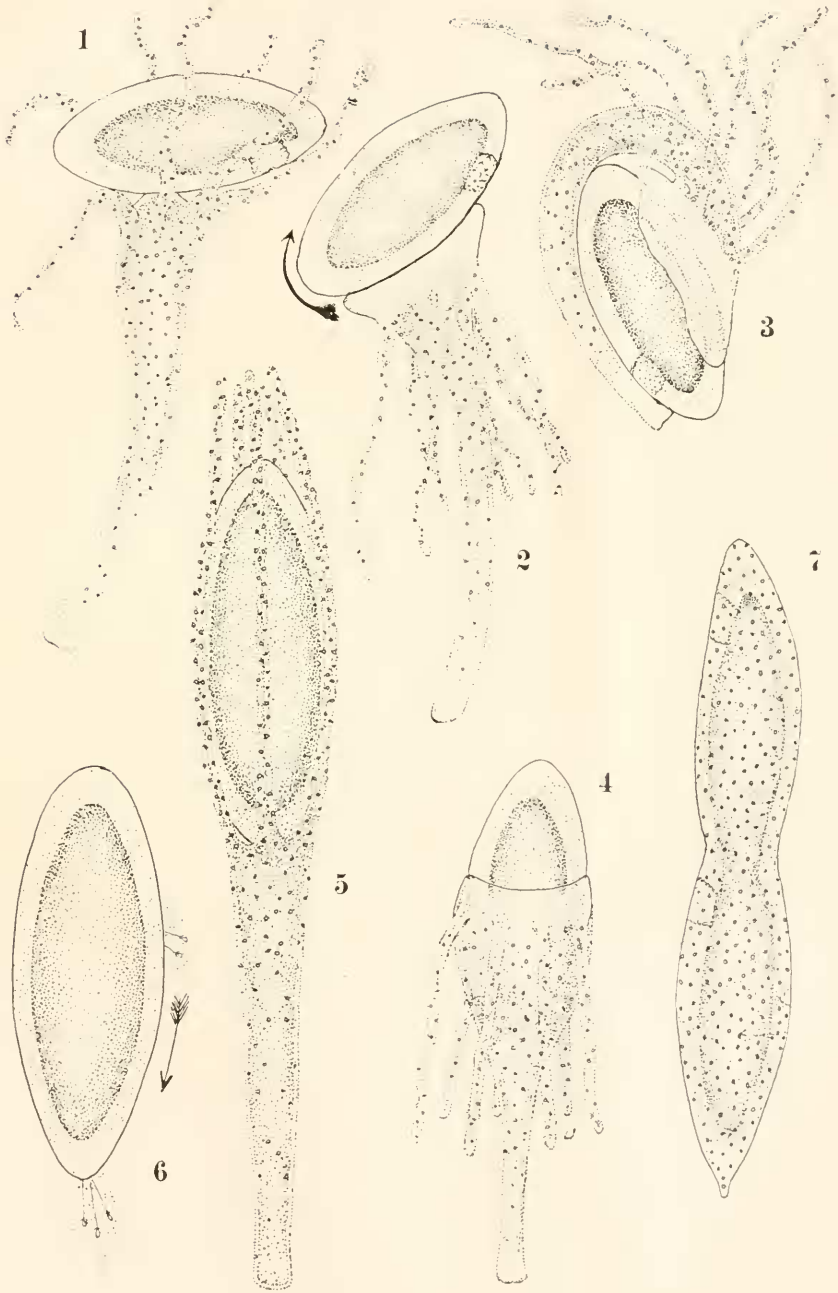
Fig. 3. The expanding peristome has glided posteriorily and dorsally, in direction indicated by arrow in Fig. 2, over surface of *Microstoma* as the *Hydra* flexed its body and pressed the *Microstoma* between its greatly expanded peristome and its bent body. From this embrace the *Microstoma* escaped. × 50.

Fig. 4. Soon after this escape the *Microstoma* returned to be captured by the tentacles of *Hydra*. After the inception of ingestion the tentacles of *Hydra* were bent back so as to lie almost parallel to the axis of the polyp. The ingestion was a slow process. × 50.

Fig. 5. Shows the manner in which a *Microstoma* was held by a *Hydra*. The *Microstoma* slowly glided out of this embrace between the ends of the tentacles. × 50.

Fig. 6. When the *Microstoma* had escaped it showed two wounded regions —one bearing two nematocysts and the other three nematocysts. These nematocysts were at first embedded so that only their poison-sacs projected from the surface of the *Microstoma*. Very early a mass of mucus formed in each wounded region. The lateral mass moved posteriorly (as shown by arrow) and fused with the posterior one. As these masses of mucus grew in length, they dragged the five stinging threads from the body of the *Microstoma*. The combined mass of mucus, containing five nematocysts, eventually was cast off. After this the *Microstoma* moved about normally. × 50.

Fig. 7. Shows a dividing *Microstoma* with a maximum charge of nematocysts at its surface. When the nematocysts are this frequent the *Microstoma* is said to be "loaded." × 50.



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SOME EFFECTS OF THE LOWER ALCOHOLS ON *PARAMECIUM*.[1]

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General—Investigators of the protozoa do not agree as to the influence of abnormal environment. For example, Matheny (1910) states that alcohol in doses of two per cent. or less "has no effect whatever" on *Paramecium,* while Calkins and Lieb (1902), and Woodruff (1908), working with doses many times more dilute report marked, but dissimilar effects.

In the present studies considerable variation in the deportment of individual paramecia from a given clone was noted, which indicates that some of the factors of error in the quantitative study of *Paramecium* are obscure, and not easy of control. As Towle (1904) observes, "The sensitiveness of paramecia for different substances varies without apparent regularity." Nevertheless it was found possible in the following experiments to obtain results of significance by counting great numbers of organisms, observing strict chemical cleanliness, and confining most of the experiments to dormant cultures of pure stocks.

Cultures—Pure lines of *Paramecium caudatum* and *Paramecium aurelia* were cultivated in battery jar infusions consisting of about 25 grams of timothy hay per liter of spring water. These were twice boiled to insure the destruction of rotifers. After about a month from the date of preparation the cultures entered upon a prolonged stage of dormancy during which little detectable change occurred until starvation was evidenced by an abrupt decline. Except where otherwise noted, only organisms from the dormant cultures were studied.

No attempt was made at bacterial control. However, in one culture a mixture of *B. lactis aerogenes* and a bacillus of the

[1] Abbreviated excerpts from an essay presented to The Johns Hopkins University in conformity with the requirements for the degree of Master of Arts (Bills, 1923*a*). A previous publication (Bills, 1923*b*) containing other excerpts should be consulted.

aquatilis group gained a long-enduring ascendency over all other bacterial forms.[2] As this culture supported the finest growth of paramecia that I have ever seen, it is interesting to note that Hargitt and Fray (1917) and Phillips (1922) maintain that simple bacterial mixtures do *not* provide as good a food for *Paramecium* as the usual complex natural mixtures.

Alcohols—The six simplest monatomic alcohols were employed: Methyl, ethyl, *n*-propyl, *i*-propyl, *n*-butyl, and *i*-butyl. All were of good purity. Dilutions in spring water were prepared volumetrically, with a micro-burette, a fresh solution being employed for each observation.

The Effect of Lethal Concentrations of the Alcohols—One clone of *P. aurelia* and two of *P. caudatum* were treated with such strengths of the four normal alcohols as sufficed to kill them in from 30 seconds to 30 minutes. The strongest concentration used was 15 per cent methyl alcohol; the weakest was 0.8 per cent butyl. All other strengths were intermediate. In about 300 individuals the process of dying was observed under a magnification of 700 diameters.

Wide variations disregarded, the phenomena usually observed follow in order: Incoördination and inactivation of body cilia; discharge of trichocysts; arrest of contractile vacuoles; modification of cyclosis in course and diminution in rate; bending of body to a crescent; convulsive rearrangement in posterior part, producing "Indian club" shape; arrest of undulating membrane in gullet; and at death, change in appearance of protoplasm to opaque and yellowish, cessation of Brownian movement, occasional formation of blisters by elevation of cuticle, and sometimes rupture of ectoplasm with discharge of endoplasm into the blisters.

Three points in blister formation warrant further mention: (1) The lower alcohols give rise to a *few* blisters which grow *rapidly,* whereas the higher ones result in *many* blisters which grow *slowly.*

(2) The existence of "susceptibility gradients" in *Paramecium* is nicely demonstrated in blister formation. Blisters

[2] I am much indebted to Dr. Percy D. Meader, of the School of Hygiene and Public Health, for the bacteriological examination of this unusual culture.

rarely, if ever, form in the oral groove, and this region is generally the last to exhibit the signs of death. But the anterior end is the most susceptible to blister formation, and it is there that the ectoplasm most frequently breaks. Furthermore, the aboral side is more susceptible than the oral, being nearly as delicate as the anterior end, in regard to both blister formation and ectoplasmic rupture. In this connection it is interesting to recall the work of Child (1914) who demonstrated in *Paramecium* anterior hypersensitiveness to cyanide.

(3) When the granules of the seemingly still living protoplasm are discharged thru the ectoplasm into a blister they do not behave precisely like free particles in a liquid; they keep together in globular masses, or in thread-like protrusions. Sometimes they may become differentiated even more distinctly from the still hyaline portion of the blister by forming a new superficial film. These observations are in accord with the researches of Seifriz (1921), who noted the tendency of living protoplasm to remain immiscible with water, and "to form, almost instantly, a membrane on its surface."

Alcohol and Resistance to Starvation—None of the earlier studies on the influence of alcohol on *Paramecium* appears to have considered the effect on starving cultures. In attacking this problem cultures were prepared by adding one volume of dormant stock culture to one volume of an alcohol of twice the desired strength. Such mixtures were apportioned in 25 cc. fractions to about 100 Stender dishes of 30 cc. capacity. Most of the dishes were kept at room temperature, and the covers removed only when observations were made at various intervals. A few of the cultures were temperature-controlled.

By a method described at length in my original essay (Bills, 1923*a*) determinations were made on the maintenance of the alcoholic content of these cultures. It was found that in spite of the closely fitting covers on the dishes the alcoholic content diminished at the rate of 21 per cent. of the original amount in five days, and 56 per cent. in 31 days, these values including loss by consumption as well as loss by evaporation. Both values are averages of 45 cultures containing 1.25 per cent. ethyl alcohol.

Three sets of observations were made. The results are recorded in Tables I., II., and III. Table I. is a record of the ac-

TABLE I.

A RECORD OF THE ACTIVITY, SIZE, AND POPULATION OF CULTURES FROM CLONE 10, UNDER THE INFLUENCE OF 1.0 PER CENT. METHYL, 0.8 PER CENT. ETHYL, 0.4 PER CENT. PROPYL, AND 0.2 PER CENT. BUTYL ALCOHOLS.

Alcohol.	Exposure.	Activity.	Size.	Population.
Methyl ..	24 Hours	Normal	Decreased	
Ethyl....		Increased	Normal	
Propyl...		Normal	Normal	
Butyl....		Increased	Normal	
Methyl ..	64 Hours	Normal	Normal	Distributed throughout culture
Ethyl....		Normal	Normal	Distributed throughout culture
Propyl...		Normal	Normal	Aggregated in dense masses
Butyl....		Normal	Normal	Distributed throughout culture
Methyl ..	91 Hours	Decreased	Decreased	
Ethyl....		Normal	Normal	
Propyl...		Decreased	Decreased	
Butyl....		Normal	Normal	
Methyl ..	25 Days		Decreased	Very few
Ethyl....			Normal	Few
Propyl...			Normal	Extremely numerous
Butyl....			Normal	Extremely numerous
Methyl ..	53 Days	Decreased		Few
Ethyl....		Decreased		Very numerous
Propyl...		Decreased		Numerous
Butyl....		Decreased		Numerous
Methyl ..	120 Days			Extinct
Ethyl....				Very few
Propyl...				Extinct
Butyl....				Extinct

tivity, size, and endurance of organisms from *Paramecium caudatum*, Clone 10 exposed for varying lengths of time to the first four normal primary alcohols in the following concentrations: 1.0 per cent. methyl; 0.8 per cent. ethyl; 0.4 per cent. propyl; 0.2 per cent. butyl. Table II. is a similar record of five discrete experiments on Clone 10 showing the difference between alchoholized and normal cultures. Controls consisting of culture diluted with an equal volume of spring water were used in this series. All the treated cultures contained 1.0 per cent. methyl alcohol. Table III. is a comprehensive record of seven cultures of paramecia,

each of which was treated with six different alcohols, and observed as to population in comparison with untreated, undiluted, controls at intervals up to two months from the time of preparation. The concentrations of methyl and *n*-propyl alcohols seem to have been a little high for some of the cultures. The alcoholic content was as follows: 2 per cent. methyl; 1 per cent. ethyl; 1/2 per cent. *n*-propyl; 1/4 per cent. *n*-butyl; 3/4 per cent. *i*-propyl; 3/8 per cent. *i*-butyl.

TABLE II.

RECORDS OF FIVE DISCRETE EXPERIMENTS ON CLONE 10 IN 1.0 PER CENT. METHYL ALCOHOL, SHOWING THE DIFFERENCES BETWEEN ALCOHOLIZED AND CONTROL CULTURES AFTER DIFFERENT PERIODS OF TIME.

1. Time, 16 days. Temperature maintained at 27.5°.
   Treated culture flourishing.
   Control beginning to starve.
2. Time, 30 days. Room temperature. Covers sealed with vaseline.
   Treated culture contains many large, slow-moving animals.
   Control died of starvation.
3. Time, 30 days. Temperature maintained at 25°.
   Treated culture contains many paramecia of almost normal size, but much vacuolated and very slow-moving.
   Control died of starvation.
4. Time, 30 days. Temperature maintained at 35°.
   Treated culture contains many small, active paramecia.
   Control died of starvation.
5. Time, 50 days. Room temperature.
   Treated culture contains many large, active, slightly vacuolated paramecia.
   Control died of starvation.

Inspection of the tables reveals that all alcohols have a similar influence on starving cultures. Not only do all of them postpone the advent of starvation, but they may even restore severely starved cultures to their former prosperity. This fact should not be taken to indicate that alcohols function *directly* as food for *Paramecium*, as they appear to do for green algæ (Moore and Webster, 1920). In the present case their mode of action is obscure. In activity the alcoholized paramecia remain normal, increase, or decrease; and in size they remain normal, or decrease—conditions attributable quite as well to nutritional as to pharmacological influence.

TABLE III.

A POPULATION RECORD OF SEVEN CULTURES EXPOSED, WITH CONTROLS, TO SIX ALCOHOLS FOR DIFFERENT PERIODS OF TIME.

The alcoholic strengths were: 2 per cent. methyl; 1 per cent. ethyl; ½ per cent. *n*-propyl; ¼ per cent. *n*-butyl; ¾ per cent. *i*-propyl; ⅜ per cent. *i*-butyl.

	Alcohol.	Population at Time of Prep.	Population 4 Days Later.	Population 30 Days Later.	Population 60 Days Later.
P. caudatum Clone 10	Control	Pale, odorless, nearly extinct from starvation.	Nearly extinct	Nearly extinct	Extinct
	Methyl		Extinct	.....	.....
	Ethyl		Multiplying	Very numerous	Excellent
	n-Propyl		Extinct	.....	.....
	n-Butyl		Slight increase	Very numerous	Numerous thin
	i-Propyl		Nearly extinct	Numerous	Extinct
	i-Butyl		Extinct	.....	.....
P. caudatum Clone 10	Control	Pale, putrid, extremely numerous. Some aggregates of paramecia have been broken up.	Excellent	Very numerous	Very few thin
	Methyl		Excellent thin	Numerous fat	Extinct
	Ethyl		Excellent	Excellent	Numerous small
	n-Propyl		Extinct	.....	.....
	n-Butyl		Excellent	Very numerous	Numerous
	i-Propyl		Numerous	Numerous	Extinct
	i-Butyl		Excellent	Numerous	Few but good
P. caudatum Clone 8	Control	Pale, odorless, dormant. Will soon be starving.	Excellent	Very few	Extinct
	Methyl		Nearly extinct	Extinct	.....
	Ethyl		Excellent	Very few	Very few
	n-Propyl		Extinct	.....	.....
	n-Butyl		Excellent	Few	Extinct
	i-Propyl		Excellent	Few	Extinct
	i-Butyl		Excellent	Very numerous	Nearly extinct
P. aurelia Clone 11	Control	Colorless, odorless, few paramecia in starvation.	Excellent	Few	Extinct
	Methyl		Few	Very few	Numerous good
	Ethyl		Excellent	Nearly extinct	Extinct
	n-Propyl		Extinct	.....	.....
	n-Butyl		Numerous	Nearly extinct	Extinct
	i-Propyl		Excellent	Extinct	.....
	i-Butyl		Very few	Extinct	.....
P. aurelia Clone 7	Control	Pale, odorless, dormant.	Numerous	Nearly extinct	Extinct
	Methyl		Few	Extinct	.....
	Ethyl		Numerous	Numerous	Accident
	n-Propyl		Extinct	.....	.....
	n-Butyl		Multiplying	Very numerous	Very few
	i-Propyl		Numerous	Few	Extinct
	i-Butyl		Numerous	Extinct	.....
P. aurelia Clone 4	Control	Pale, odorless. Few paramecia, somewhat starved.	Numerous	Very numerous	Few thin
	Methyl		Numerous	Very numerous	Few good
	Ethyl		Multiplying	Nearly extinct	Few thin
	n-Propyl		Extinct	.....	.....
	n-Butyl		Numerous	Nearly extinct	Numerous
	i-Propyl		Numerous	Numerous	Accident
	i-Butyl		Very few	Numerous	Numerous
P. caudatum Wild culture	Control	Colorless, putrid. The aggregates of paramecia have been well broken up. Extremely numerous.	Extinct	.....	.....
	Methyl		Extinct	.....	.....
	Ethyl		Few	Excellent	Numerous thin
	n-Propyl		Few	Very numerous	Numerous small
	n-Butyl		Very numerous	Few thin	Nearly extinct
	i-Propyl		Few	Very few	Few thin
	i-Butyl		Numerous	Excellent	Numerous thin

The Influence of Temperature on the Susceptibility of Paramecium to Ethyl Alcohol.—Within reasonable limits of constancy a given concentration of a given alcohol will narcotize a definite per cent. of the organisms in a particular culture in one hour. This fact makes it possible to compare quantitatively the narcotic action of various alcohols, and to study the modifying influence of physical conditions on the effects of a particular alcohol. The method devised for counting the paramecia "narcotized" and those "unaffected" is elsewhere described (Bills, 1923*b*).

In the present experiment counts were made at widely different temperatures—8° and 25°. *Paramecium caudatum,* Clone 10, and 3.0 per cent. ethyl alcohol were used, and all observations made in duplicate. From the data presented in Table IV. it appears that the per cent. of the paramecia narcotized at 8° does not differ significantly from the per cent. at 25°. It seems improbable (though of course possible) that intermediate temperatures would show any markedly different values.

TABLE IV.

SHOWING THE INFLUENCE OF TEMPERATURE ON THE SUSCEPTIBILITY OF PARAMECIA TO ETHYL ALCOHOL.

The experiments were conducted in darkness.

Temperature.	Number of Paramecia Narcotized.	Number of Paramecia Unaffected.	Per Cent. of Paramecia Narcotized.
8°	50	793	5.9
8°	43	473	8.3
25°	47	568	7.6
25°	32	358	8.2

Average per cent. narcotized at 8° = 7.1.
Average per cent. narcotized at 25° = 7.9.

The Influence of Light on the Susceptibility of Paramecium to Ethyl Alcohol.—Pairs of burettes containing paramecia of Clone 10, with and without 3.0 per cent. ethyl alcohol were kept for one hour in strong, but diffuse, northern, daylight, or in direct, brilliant, sunlight in the middle of April. The direct light passed obliquely through the thin glass walls of the burettes. In all experiments the temperature was between 21° and 23°.

The data are presented in Table V. This table shows that direct sunlight inactivated in one hour 28 per cent. of the organ-

isms in plain spring water, but that under like conditions, except that 3.0 per cent. ethyl alcohol was present, the sunlight inactivated 42 per cent. The experiments were repeated in diffuse daylight, and not one individual was inactivated in the absence of alcohol, while with 3.0 per cent. alcohol 8.9 per cent. were affected—a figure not significantly different from the values got in the temperature experiments which were made in darkness.

TABLE V.

SHOWING THE INFLUENCE OF LIGHT ON THE SUSCEPTIBILITY OF PARAMECIA TO ETHYL ALCOHOL.

Light.	Per Cent. Alcohol.	Number of Paramecia Narcotized.	Number of Paramecia Unaffected.	Per Cent. of Paramecia Narcotized.	Average.
Diffuse daylight....	0.0	0	1361	0.0	0.0
Diffuse daylight....	3.0	213	1641	12	
Diffuse daylight....	3.0	28	454	5.8	8.9
Direct sunlight.....	0.0	558	1432	28	
Direct sunlight.....	0.0	451	1150	28	28
Direct sunlight.....	3.0	888	914	49	
Direct sunlight.....	3.0	726	1306	35	42

The Combined Effect of Preliminary Aëration and Agitation of a Paramecium Culture on its Subsequent Susceptibility to an Alcohol.—Aëration was effected by agitating for two minutes some paramecia of Clone 10 in 1.6 per cent. of *i*-propyl alcohol. This alcohol was chosen because of the fine froth produced when cultures containing it are violently shaken. The results presented in Table VI. show that the aërated paramecia are decidedly less susceptible than normal controls. Of the agitated organisms 17 per cent. were narcotized, whereas 32 per cent. were narcotized in the non-aërated control culture.

TABLE VI.

SHOWING THE INFLUENCE OF AËRATION AND AGITATION ON THE SUSCEPTIBILITY OF PARAMECIA TO 1.6 PER CENT. *i*-PROPYL ALCOHOL.

Treatment of Culture.	Number of Paramecia Narcotized.	Number of Paramecia Unaffected.	Per Cent. of Paramecia Narcotized.
Agitated and aërated....	239	1139	17
Normal control.....	682	1460	32

The Question of the Adaptation of Parmecium to Alcohol.— How does a preliminary exposure of *Paramecium* to a low concentration of ethyl alcohol affect the subsequent resistance to a stronger dose of ethyl alcohol, and of its homologues?

Towle (1904), working with electrolytes and simple organic compounds, concluded that "paramecia become readily habituated to solutions in strengths which are not soon fatal." Daniel (1908) found that paramecia when transferred *gradually* into distilled water become adjusted to this otherwise deadly substance. Estabrook (1910) developed in *Paramecium* a temporarily increased tolerance for strong doses of sodium chloride. Neuschlosz (1921*a*) found that *Paramecium* can develop a high resistance to dyes of the thiazin, benzidin, and triphenylmethane series. Neuschlosz later (1921*b*) reported that paramecia acclimatized to trivalent arsenic are at the same time resistant to trivalent antimony. Woodruff (1908) observed that alcoholized paramecia become more sensitive to copper sulphate. Their behavior toward a stronger dose of *alcohol* was not recorded. A case of adaptation in *Spirostomum* and *Stentor* reported by Daniel (1909) is of special interest, inasmuch as the method of experimentation is essentially identical with my method on *Paramecium;* the results, however, being different from mine. Daniel claims that he sometimes produced in these protozoa a slight adaptation to ethyl alcohol, but that this was accompanied by an increased susceptibility to methyl alcohol.

My experiments were made as follows: To 10 cc. of the Clone 10 culture taken from near the surface 10 cc. of 2.0 per cent. ethyl alcohol was added, making a 1.0 per cent. solution of alcohol. This mixture was put into a 30 cc. Stender dish and kept at approximately 24° for three days. At the end of this period the paramecia were observed to be distributed thruout the medium, appearing healthy, and distinctly more active than the controls altho possibly somewhat thinner. They were then exposed for one hour to each of the six alcohols in the concentrations indicated in Table VII., using quantities large enough to eliminate practically all error resulting from the presence of the original ethyl alcohol (see Bills, 1923*a*).

The results obtained are presented in Table VII. In this

table the values given for the untreated controls are interpolated averages obtained for the six alcohols in a series of experiments elsewhere described (Bills, 1923*b*). They were made a few days before the present experiment was performed, during a period of extended constancy in the cultures. Therefore, these values are admissible for comparison here, and are probably more nearly accurate than single observations would have been.

From Table VII. it is clear that the three-day exposure to 1.0 per cent. ethyl alcohol increased the susceptibility of the paramecia to a narcotizing concentration of ethyl alcohol; and, similarly, to each of the other five alcohols. In other words, paramecia are not acclimatized to ethyl alcohol under the conditions of this experiment. Unlike Daniel's spirostoma and stentors which under similar conditions became more resistant to ethyl alcohol, paramecia became more susceptible to *all* alcohols.

TABLE VII.

SHOWING THE EFFECT OF EXPOSURE TO ALCOHOL ON THE ACTION OF ALCOHOLS ON *Paramecium*.

Period of Acclimatization to 1.0 Per Cent. Ethyl Alcohol:	Narcotizing Alcohols.	Per Cent. of Treated Paramecia Narcotized.	Per Cent. of Untreated Paramecia Narcotized.
72 hours.....	Methyl, 5.0%	Mostly disintegrated	30
72 hours.....	Ethyl, 3.3%	Many disintegrated	24
77 hours.....	*n*-Propyl, 0.9%	35	31
77 hours.....	*n*-Butyl, 0.5%	44	31
79 hours.....	*i*-Propyl, 1.6%	55	29
79 hours.....	*i*-Butyl, 0.4%	54	35

I wish to express my appreciation of the interest and guidance given me in the course of these experiments by Professor S. O. Mast and Professor H. S. Jennings; and my thanks to many other persons for their assistance in many ways.

SUMMARY.

1. A mixture of *B. lactis aerogenes* and *B. aquatilis* (sp. ?) constitutes the best food found for *Paramecium*.

2. When paramecia are exposed to an alcohol in sufficient strength they are at first incoördinated in movement and then inactivated. Later toxic effects are manifested by marked in-

ternal changes, formation of "blisters" by elevation of cuticle, rupture of ectoplasm, and death.

3. The anterior end of *Paramecium* is more susceptible to alcohol than the posterior end, and the aboral side more than the oral.

4. Indirect daylight has no perceptible effect on normal or alcoholized paramecia, but direct sunlight inactivates them; this effect is augmented in the presence of alcohol.

5. Change in temperature over a wide range has no appreciable effect on the susceptibility of paramecia to alcohol.

6. Aëration and agitation of a *Paramecium* culture renders the paramecia much less susceptible to alcohol.

7. Paramecia in a given solution without food live longer with alcohol than without; starving cultures can even be restored to prosperity by the addition of suitable amounts of any alcohol.

8. Exposure of paramecia to weak ethyl alcohol increases their susceptibility to a stronger dose of ethyl alcohol, and to five other alcohols.

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BIOLOGICAL BULLETIN

REACTIONS OF THE LARVÆ OF THE SHRIMP, *PALÆMONETES VULGARIS*, AND THE SQUID, *LOLIGO PEALII*, TO MONOCHROMATIC LIGHT.

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Since the larvæ of the shrimp, *Palæmonetes vulgaris*, and the squid, *Loligo pealii*, react to light very positively, it seemed interesting to discover what portion of the spectrum is most effective in stimulating them. For this purpose experiments were performed at the laboratory of the U. S. Fish Commission during the summers of 1920 and 1921 and at the Marine Biological Laboratory, Woods Hole, Mass., in the summer of 1923. The writer is indebted to Dr. S. O. Mast for valuable suggestions and criticism.

METHOD.

It was found that when the larvæ of the shrimp, *Palæmonetes vulgaris*, and the squid, *Loligo pealii*, are exposed to light, they turn and move definitely in the direction of its source. When exposed in a square aquarium at the intersection of two beams of light of equal intensity and at right angles to each other, the larvæ tend to distribute themselves in approximately equal numbers on the two sides of the aquarium which are most highly illuminated. If, however, the light in one of the beams is of greater intensity than that in the other, more larvæ aggregate on the side of the aquarium toward the brighter light. In other words the larvæ act as a sort of living photometer. It seemed possible, therefore, to apply to these organisms the method described by Mast (1907, 1917) for testing the relative stimulating efficiency of light of various wave-lengths.

The larvæ were placed in a small square glass container (5 cm. x 5 cm. x 2.5 cm.); opposite one side was placed a 10-watt Mazda lamp in a light-tight box (5.5 cm. x 5.5 cm. x 9 cm.) with an opening on the side toward the aquarium. The opening was covered by a smoked glass which reduced the illumination to 4.4 candle power. This box was movable on a meter scale toward and away from the container. Opposite an adjoining side of the aquarium at right angles to this was another box (5 cm. x 5 cm. x 14 cm.) containing a 15-watt Mazda candelabra lamp, in front of

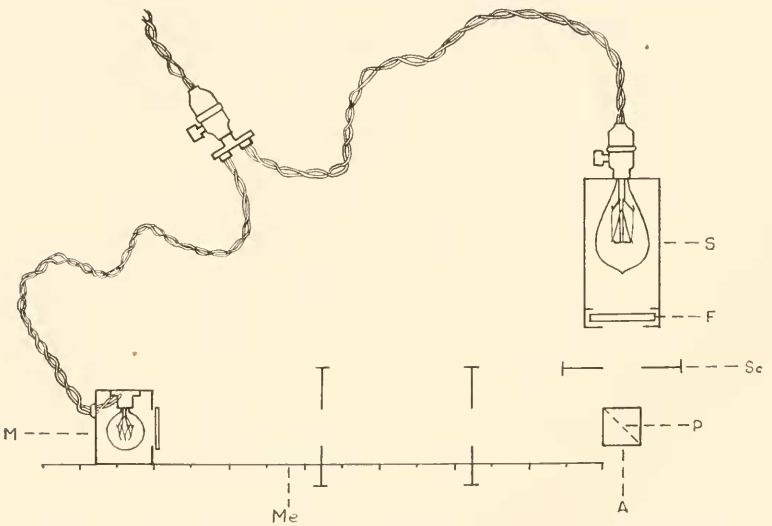

FIG. 1. Diagram of apparatus. *A*, aquarium; *F*, Wratten light filter; *M*, movable lamp; *Me*, meter stick; *P*, removable partition; *S*, stationary lamp; *Sc*, opaque screens.

which could be inserted monochromatic light filters; this box was in a fixed position with the light 10 centimeters from the aquarium. Both lamps were operated by a single switch so that the larvæ could be exposed simultaneously to light from the two sources. The lamp producing white light was moved back and forth on the meter scale until the point was found at which the larvæ distributed themselves equally with respect to the two sources of light. The white and the colored lights were then considered to be of equal stimulating effect. Black screens with openings somewhat larger than the source of light were so arranged as to minimize reflected light (Fig. 1).

Monochromatic light was secured by means of Wratten Light Filters Nos. 70, 71, 72, 74, 75, 76, and a combination filter composed of Nos. 22 and 53 made by Eastman Kodak Company. Each filter transmits a rather narrow band of the visible spectrum, most of the light being limited to a band 40 $\mu\mu$ wide or less. The per cent. of incident light of various wave-lengths transmitted by these filters has been measured by Eastman Kodak Company and is represented graphically by the white areas in Fig. 2.

The distribution of energy in the spectrum of the 15-watt Mazda lamp used in connection with the filters was ascertained by the U. S. Bureau of Standards (Fig. 3).

Since the distribution of the energy incident on the filters (Fig. 3) and the portion of this energy transmitted by each (Fig. 2) was known, and both had been ascertained for bands 10 $\mu\mu$ wide, it was possible to compute the relation in the energy of the light transmitted by the different filters in conjunction with the lamp in 10 $\mu\mu$ bands (Table I.).

TABLE I.

RELATIVE ENERGY TRANSMITTED BY DIFFERENT WRATTEN LIGHT FILTERS WITH 15-WATT MAZDA LAMP.

Filter Number.	Relative Energy.
70 (red)	1176.0
71 (red)	225.0
72 (orange)	74.0
22 and 53 (yellow)	38.5
74 (green)	39.3
75 (green)	51.8
76 (blue)	10.35

In addition to the transmission in the visible spectrum all the filters transmit infra-red, but these rays were found to be ineffective for the larvæ of both *Palæmonetes vulgaris* and *Loligo pealii*. This was determined by subjecting them to light from which nearly all the visible rays were removed by means of Wratten Light Filter No. 88 which transmits only rays beyond 700 $\mu\mu$. Since the infra-red rays proved to be ineffective in stimulating the larvæ, they may be left out of consideration.

Ultra-violet was screened out by several layers of glass through which the light passed.

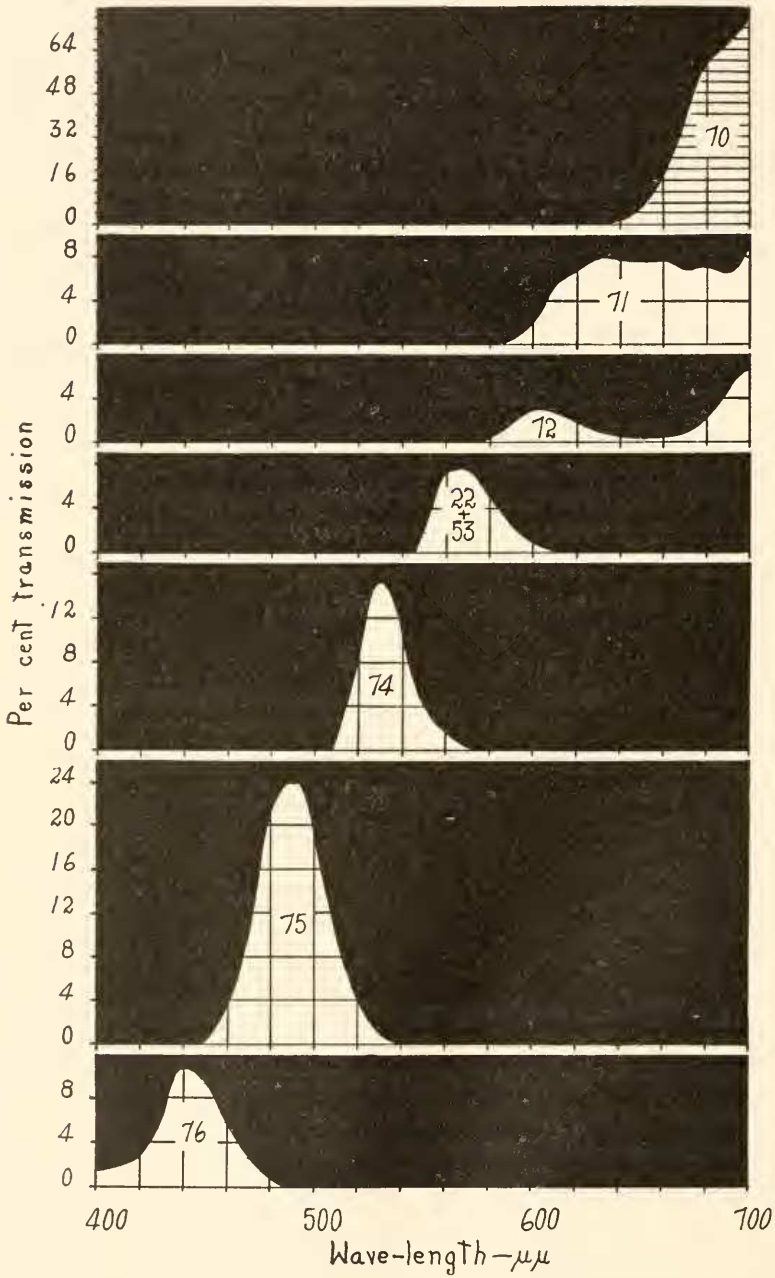

FIG. 2. Per cent. of light transmitted by Wratten filters used to secure monochromatic illumination.

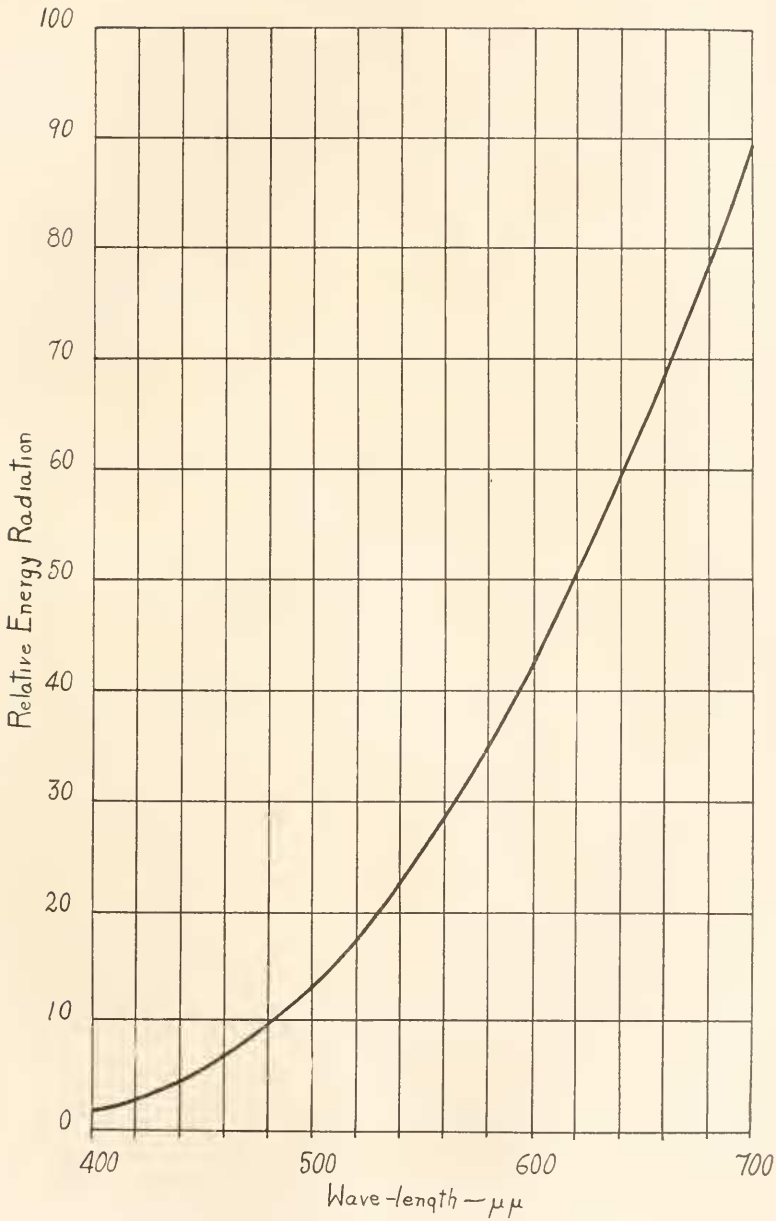

FIG. 3. Distribution of energy in the spectrum of the 15 watt Mazda lamp used, operated at 105 volts.

Experiments.

For both the shrimp and squid larvæ the experiment was performed as follows: A known number of larvæ, usually about thirty-five, were inserted into a small glass tube standing upright in the corner of the aquarium where the rays intersected, and allowed to remain one half minute in order to become dark-adapted. Both lights were then turned on simultaneously, and the glass tube carefully lifted out so as to free the larvæ without producing currents in the water. The larvæ were exposed for one minute, at the end of which interval a partition was slipped down into the aquarium in such a way as to separate the halves facing the two sources of light. The larvæ in each half were then counted and the number recorded; the light which had attracted the more larvæ was considered the more effective. In this manner for each filter numerous readings were taken with the movable lamp at various distances from the container, until the point was found at which the larvæ were equally distributed with respect to the two lights. At this point the stimulating effect of the light from the two lamps was, as previously stated, equal. The filters were found to differ in their effect in stimulating both the shrimp and squid larvæ, that is, for certain filters the movable lamp had to be placed nearer to the aquarium than for others, in order to obtain an equal distribution of the larvæ in the two halves of the container. Table II. and Fig. 4 summarize the results obtained.

Results and Conclusions.

In this table is shown the relative stimulation of the light transmitted by the different filters. Since the stimulating effect of the light transmitted by each filter is equal to the light produced by the movable lamp when this is so placed that the larvæ distribute themselves in equal numbers in the two halves of the aquarium facing the two sources of light, the relative stimulation of light transmitted by the different filters can be expressed in terms of the intensity of the light produced by the movable lamp. This is inversely proportional to the square of the distance of the movable lamp from the aquarium. These values are given in Table II. In order to translate these values on to the basis of relative energy, red filter no. 70 was taken as unity with a

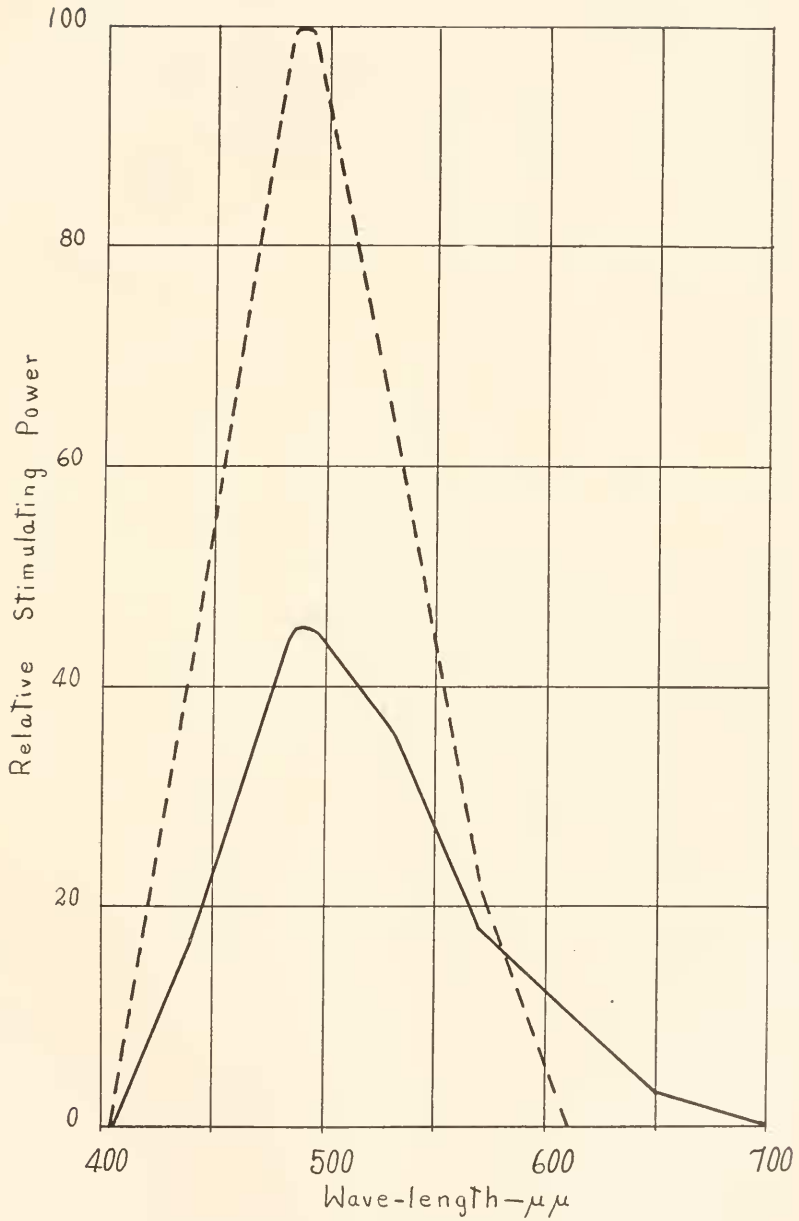

FIG. 4. Distribution of stimulating efficiency in the spectrum for shrimp and squid larvæ. Solid line, shrimp larvæ; broken line, squid larvæ.

relative energy value of 1176 (Table I.), and this number was divided by the relative energy value for each of the other filters. The resulting quotient in the case of each filter was then multiplied by the value for the relative stimulating effect of the light for that filter, thus giving values for the relative stimulating effect of light of equal energy content.

TABLE II.

RELATIVE STIMULATION OF LIGHT TRANSMITTED BY DIFFERENT FILTERS.

Wratten Filters.	Shrimp Larvæ.		Squid Larvæ.	
	Illumination.	Corrected For Energy.	Illumination.	Corrected For Energy.
Red No. 70	4.95	4.95	0	0
Red No. 71	2.77	14.46	0	0
Orange No. 72	1.38	24.88	0	0
Yellow Nos. 22 and 53	3.3	100.65	4.0	122.0
Green No. 74	6.25	201.25	11.11	356.63
Green No. 75	11.11	252.2	25.0	560.0
Blue No. 76	.83	94.29	2.04	231.74

The results obtained (Table II. and Fig. 4) show that for both the shrimp and squid larvæ the maximum stimulation is in the blue-green (470 $\mu\mu$–510 $\mu\mu$), and that yellow-green (520 $\mu\mu$–550 $\mu\mu$), yellow (550 $\mu\mu$–590 $\mu\mu$), and blue (420 $\mu\mu$–470 $\mu\mu$) are less stimulating. All these wavelengths are more stimulating for squid than for shrimp larvæ, particularly the blue-green and blue which are more than twice as effective. The rays beyond 620 $\mu\mu$ do not seem to effect squid larvæ at all, while shrimp larvæ, though not so powerfully stimulated, show some reaction.

Since both shrimp and squid larvæ possess eyes, it is reasonable to suppose that their reactions are due, to a large extent at least, to the nature of the photosensitive substances present in the eyes. Whether this is associated at all with the fact that the shrimp eye is compound while the squid has camera eyes would be interesting to learn.

Mast (1917) found from the study of fifteen species of various types that stimulation by light depended upon the wave-length. The distribution of stimulating efficiency in the spectrum differed in various forms without any apparent relation as to whether they were closely related species or not. It may be

noted that in the majority of cases the wave-lengths which he found to be most stimulating were among those transmitted by the blue-green Wratten Light Filter No. 75, which was most stimulating for *Palæmonetes* and *Loligo*. The blue-green region was also found to be maximum in stimulating efficiency for a considerable number of different forms by Strasburger (1878), Engleman (1882), Verworn (1889), Wilson (1891), Gross (1913), Loeb and Wasteneys (1916), Laurens and Hooker (1920), Hecht (1921), and others. Various longer waves were, however, found to be most efficient in other species by Bert (1869), Lubbock (1991), Engelman (1882), Hess (1910), Loeb and Maxwell (1910), and others. It would appear, therefore, that the distribution of stimulating efficiency in the spectrum is not necessarily the same for all species having light-perceptive powers.

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FERTILIZATION, CORTEX, AND VOLUME.

OTTO GLASER.

I.

The initiation of development is associated with alterations in the cortex of the egg. Some of these changes are visible under the microscope; others are matters of inference. But writers on the mechanism of fertilization by no means agree in their interpretation of either the observed or the postulated phenomena. Indeed, there is uncertainty even in the realm of direct observation. For example, the fertilization membrane is considered by some as an epigenetic structure formed at the moment of impregnation, whereas others claim that fertilization merely results in changes by which a preformed membrane is rendered visible. Again there is the question of volumes. In 1913 and '14, I claimed for *Arbacia* as well as *Asterias*, an immediate loss of volume at fertilization; in 1921 Chambers claimed a constant volume and even an actual increase.

In themselves these details do not appear very important. It is not obvious just how current interpretations would be affected if it were possible to convince everyone either that the fertilization membrane is preëxistent or formed de novo. Yet with respect to volume, the case has practical consequences. It is true that here too, no compulsion, theoretical in origin, or otherwise, forces us to favor any particular assumption. Whether the egg increases, decreases, or remains constant does not inevitably involve any revision in theory. Of course, a decrease in volume as well as an increase might result from individual one-way mechanisms different in the two cases, whereas a constant volume might merely signify the absence of relevant changes of any sort. On the other hand, each of the three possibilities is conceivable as the resultant of antagonistic processes, one or more of which could be common to all. This, however, by no means implies that it is immaterial, practically, which of the three things is true. In our present state of knowledge, one of the three possibilities, it happens, is capable of making a more significant comment than

the other two. For this reason, then, it is worthwhile to re-examine the question and to decide if possible whether the egg increases in volume, decreases, or remains constant.

II.

The question, unfortunately, has entanglements. Historically, as well as through needless misunderstandings, it is bound up with the problem of the fertilization membrane. This structure, despite the very clever criticisms recently made by Garrey ('19[1]) can be demonstrated on the surface of the unfertilized egg. After fertilization, the membrane demonstrates itself—a change brought about by easy stages in certain eggs.

In *Asterias*, for instance, the membrane, at ordinary temperatures, becomes visible rather slowly. In optical section, the perivitelline space is noticeable first as a small clear crescent at one point on the surface of the egg. From this region of initial visibility, the narrow area spreads in both directions until the horns of the crescent have met and completed approximately a circle. At this moment the egg is no longer in direct contact with the membrane whose diameter is now patently greater than before.

All this was implicit in my earlier statement ('13) in which I wrote: "the egg peels itself away from the inner surface of a thin preëxisting membrane. This peeling away seems to depend not upon changes in the fertilization membrane but upon changes in the surface film of the egg. When this is rendered more permeable, material leaves the egg and the egg shrinks away from its closely adherent covering which then becomes visible."

I applied the same interpretation also to the *Arbacia* egg though its perivitelline space is very much smaller. Quite recently the process of peeling has been very clearly described in *Echinarchnius* by Just ('19[2]). Chambers ('21) on the contrary, seems to have misunderstood my meaning. I did associate a shrinkage of the egg with fertilization and the appearance of the membrane; however I did not claim for either sea-urchin or starfish an exclusive monopoly of shrinkage or that "the initial diameter of the completed fertilization membrane is equal to that of the unfertilized egg" (*loc. cit.*, . . ., p. 332). Indeed, I can discover no relation between this statement and the facts, or my descrip-

tion of them. Material leaves the egg and the egg shrinks. These two processes appeared to me as intimately linked and for part of the time simultaneous. Furthermore I discussed the material which the egg eliminates ('14, p. 91). This secretion or excretion, as Loeb ('08) long ago rendered probable, contains a colloid to which the fertilization membrane itself is impermeable. The substance in question completes the conditions under which the perivitelline space can exert an osmotic pressure greater than that of the sea-water outside. Accordingly the membrane must become distended and its actual distension is so clear optically that no one before has ever doubted it.

Summarizing, we may say: the egg shrinks away from its enclosing membrane and the membrane becomes distended. These two movements in opposite directions account for the existence and volume of the perivitelline space.

III.

But does the egg actually shrink? Chambers denies the fact. The *Asterias* egg, he claims, gives no indication of volumetric decrease. If one waits long enough there is a measurable increase which I failed to notice because my "measurements were made on the assumption that the eggs always maintain a spherical shape." However the *Asterias* ovum is "very soft and if allowed to lie on the bottom of a glass dish tends to flatten into the shape of a disc," which "upon fertilization . . . rounds up as the fertilization membrane leaves its surface" (*loc. cit.*, . . ., p. 332). Under these conditions, observations taken in one plane would inevitably lead to erroneous conclusions—a deduction which is logically incontestible; which applies without question to the starfish egg at certain times and yet whose inapplicability to my work on *Arbacia* can be expressed quantitatively as 98.5 per cent.

For the sake of the discussion, I will assume that the eggs with which I worked were somewhat distorted by weight. It nevertheless remains true that in *Arbacia* gravity and surface forces quickly establish an equilibrium in which the unfertilized egg can maintain itself without appreciable change, for at least four hours ('14). During this time further movement toward or away from the spherical condition is negligible.

Under similar conditions the *Asterias* ovum changes markedly. The freshly shed egg is often pear-shaped and frequently flattened. It approaches the spheroid condition in the course of an hour in sea-water and it is then possible to select eggs not distinguishable from spheres. It was with such that I worked; they had established equilibrium and consequently were treated exactly like those of *Arbacia*. But in order to be consistent, I must assume that here again there was some flattening by weight and so for both sorts of eggs the question to be answered is the same: How much of the apparent decrease in diameter is the result of a change in shape and how much is indicative of an actual loss in volume?

The magnitude of the loss which I reported for Asterias is much greater than that found in *Arbacia*. However, all except twenty-five of my original measurements were based on the sea-urchin egg. In my search for further evidence, I therefore limited myself entirely to the second type.

The methods employed differed somewhat from the technique of Chambers. As before I limited myself to the period of actual fertilization. In these newer measurements I again preferred the stage micrometer to one of the ocular type. I also did not pierce the chorion with a needle or roll my eggs on gelatin plates in order to remove the jelly. In the absence of proof to the contrary, these procedures cannot be considered innocuous on account of the high salt content of the chorion ('22). In fact I used the chorion in order to avoid all possibility of discoidal distortion. This was accomplished by entangling the jelly on the minute fibrils of a slightly roughened cotton thread. In this way it is easily possible to prepare depression slides on which a considerable number of eggs have been ensnared by several fibers each one sticking to the jelly at a single point. This method has several advantages; the eggs remain "afloat" and move in the liquid whenever the fibrils bend; without being in contact with a solid surface, every egg if desired can be identified by its position along the thread; the attachment of the fibers at several points in the chorion corrects any distortion that might result from suspension at only one point. Furthermore the eggs in these preparations can be made to yield accurate records of volume and shape by projection in the form of silhouettes upon

sensitized paper. The diameters of such prints can be measured by means of fine calipers and a vernier scale; while the exact magnification is made known by a similar projection of the scale of a stage micrometer. With the data thus secured the actual diameters of the eggs can be easily computed.[1]

In the following table (Table I.) no attempt has been made at the identification of individuals. The experiment is based on twenty-two eggs photographed before fertilization and again immediately after insemination.

TABLE I.

DIAMETERS OF EGGS.

Unfertilized.	Fertilized.
71.6 μ	74.8 μ
75.4	74.6
76.3	72.9
75.6	61.4
72.6	69.9
77.4	72.9
78.4	71.3
76.9	74.0
78.6	71.6
65.9	74.3
74.4	73.3
75.4	72.0
75.4	69.5
75.4	76.0
71.9	72.3
77.1	70.3
76.3	73.1
72.0	70.8
75.6	69.5
74.1	70.8
71.2	77.5
73.0	74.5
Average: 74.6	*Average:* 72.1

The result here—a loss of 2.5 μ is the same in sense as that found in a comparable mass experiment reported in 1914.

I next repeated the test on a smaller number of eggs whose identity as individuals, before and after fertilization was guaranteed by their position along the threads. The results are assembled in Table II.

[1] For help in making these projections under the most favorable conditions, I am greatly indebted to Dr. Selig Hecht, who suggested the use of sensitized paper. Before I made the final measurements Dr. Hecht codified the prints and until I had his key to the system of labelling I could not by any means identify corresponding silhouettes. The measurements of the eggs therefore were made under circumstances which rendered the personal equation negligible.

TABLE II.

DIAMETERS.

Series.	Egg.	Unfertilized.	Fertilized.
A	1	74.1 μ	71.8 μ
	2	74.8	74.2
	3	73.3	71.4
	4	75.6	74.6
B	1	77.0	74.6
	2	75.8	73.7
	3	80.5	78.0
	4	78.2	72.9
	5	76.5	72.5
C	1	74.6	73.0
	2	72.9	72.2
	Average:	*75.7*	*73.5*

Here again the outcome is a loss of 2.2 μ.

This may be said to complete the crude verification of my earlier results. However there are suggestive discrepancies and this remains true whether we compare the mass experiments or those in which the eggs were kept identified throughout. Thus:

	1914.		1922.	
	Mass Experiment.	Individual Eggs.	Mass Experiment.	Individual Eggs.
Average Loss.....	3.4 μ	4.08 μ	2.5 μ	2.2 μ
Corrected.....		.. 3.5		
Total Average Loss		3.45 μ		2.35 μ

In this tabulation, I have averaged all the observations, but have introduced one correction in those of 1914. My original list of individual eggs, it happens, includes one in which the recorded loss was 11.8 μ—a figure which is probably wrong. Yet this elimination makes no essential difference. An absolute discrepancy in the magnitude of the losses reported now and in 1914 remains. This error, referred to the diameters of the original eggs, amounts to 1.5 per cent. Is this significant? Does it mean that the eggs of 1914 were to this extent flattened?

IV.

If so, it should be possible also to verify the results of Chambers provided only we can make the necessary observations under

strictly comparable conditions. It will be recalled that Chambers "pierced the surrounding jelly with a needle." He did this in order that "the egg to be measured could be held suspended in the middle of the drop," hanging by one point from the ceiling of his moist chamber. Inasmuch as the starfish egg is "soft;" the jelly attached to the vitelline membrane; and this, prior to fertilization, to the plasma surface, the egg if distorted would, of course, approach a cylindrical form. Under the conditions described by Chambers, the observer would look lengthwise along the cylinder, and since the egg "rounds up" on fertilization, it follows that the initial measurement might be the diameter of a cylindroid and the final one that of a sphere. The second value should be greater than the first. But Chambers reports an immediate equality. One of two things then must have been true: either his eggs were not distorted—or the effect on the diameters produced by the change in shape was compensated by a loss in volume.

What is the likelihood that his eggs were distorted? It seems to me considerable for even the *Arbacia* egg under similar circumstances gives no indication of a decrease in diameter.

For these particular observations I selected eggs suspended from a single point. The measurements therefore were made under the conditions stipulated by Chambers. If now there is cylindroid distortion before fertilization, the "rounding up" process—which incidentally the *Arbacia* egg also exhibits—should give us a second measurement no smaller and possibly even larger than the first. The values recorded in Table III. speak for themselves.

TABLE III.

DIAMETERS OF EGGS.

Suspended at One Point.

Egg.	Unfertilized.	Fertilized.
1	76.3 μ	76.9 μ
2	72.3	72.5
3	75.7	76.0
4	73.5	74.2
5	72.9	74.2
6	74.4	74.2
Average:	74.2 μ	74.7 μ

V.

In accordance with these results then, the *Arbacia* egg by its own weight, is capable of flattening in sea-water. This vindicates the point made by Chambers. However, the extent of this flattening is negligible for the issues here at stake. This is shown by the measurements made under circumstances that leave no room for discoidal deformation. On the other hand, cylindroid distortion, when possible, may completely mask any losses in spherical volume.

We must now ask whether the loss here postulated anew for the *Arbacia* egg, is unique and whether it articulates well with the other facts of fertilization.

I did not repeat the work on the starfish because the difference there between the unfertilized and fertilized egg is even greater than in *Arbacia*. For *Nereis* too, special measurements, merely for the purpose of demonstrating a decrease in volume seem superfluous because the elimination of a voluminous mass of visible jelly is sufficient evidence. Again, according to Okkelberg ('14[2]) the egg of the brook lamprey is subject to a similar loss on fertilization.

These cases, of course, do not imply that losses everywhere must remain uncompensated long enough for us to measure them. In certain instances, alterations in shape may offset actual decreases in diameter; in others there may be compensatory swelling due to osmotic changes. Indeed, ultimately and no matter what may be true at the moment of fertilization, the egg increases in volume. The measurements which Chambers made 10, 20, and even 70, minutes after impregnation have no bearing on the immediate reorganizations of fertilization, however pertinent they may be in a study on growth.

But how do these losses in volume fit in with other facts falling within the fertilization period? In an earlier paper on egg-secretions ('14[3]) I compared the rates at which unfertilized *Arbacia* eggs and eggs in process of fertilization discolor the sea-water. The rates I found to be related as 2:3. During the summer of 1922 I repeated these observations in another way. On each of two small identical filters, I placed 1 cc. of a certain preparation of dry shed eggs. To the one filter was added a drop of sea-water; to the other a drop of dry sperm. The contents of

the two filters were then gently mixed with a blunt glass rod, thus insuring a uniform distribution, in the one case of eggs and sperm; in the other of eggs and sea-water.

Although the experiment gave the expected result, it was not possible in this way to measure the loss of material from the eggs undergoing fertilization. For this, the filter papers and the capillary spaces between the papers and the funnels held back too much liquid. Nevertheless it was very evident that the amount of fluid secreted under these conditions by the inseminated eggs was markedly greater than that produced in the same time by the controls.

Evidently something leaves the eggs as they are undergoing fertilization—a fact which relates very easily to the observed decreases in diameter. In their turn these harmonize well with the observed cortical changes—themselves the basis of postulated and reasonably well attested increases in permeability. Indeed, we have here a system of closely interdependent events and for that reason I consider the loss in volume a matter of sufficient theoretical interest to warrant the expenditure of great care in those cases in which its demonstration is possible.

This opportunity the Arbacia egg seems to offer. The decrease in its diameter is not an illusion but the index of a loss in volume correlated with a real loss in mass; it also is not an oddity. Other eggs exhibit the same phenomenon. Furthermore the fact itself is not unintelligible. Indeed it may be related significantly and without violence to the entire constellation of events which together make up the initiation of development.

AMHERST COLLEGE,
November 25, 1923.

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VITAL STAINING OF AMŒBOCYTE TISSUE OF LIMULUS.[1]

LEO LOEB AND KENNETH C. BLANCHARD.

In our analysis of the factors underlying tissue formation,[2] it was found necessary to make use of vital staining in order to elucidate certain effects of environmental conditions on the cells and on amoeboid movement.

In the course of these investigations we made some observations on the staining of the amœbocyte; these observations may contribute to the understanding of the manner in which vital stains enter cells and are fixed or held back within the cells.

1. *Neutral Red.*—Two methods were used: (1) In the first one we prepared amœbocyte tissue in small stender dishes and replaced the supernatant serum by a solution of neutral red in a *n*/2 NaCl solution of the strength of 1:4000. After the tissue had been acted on by the staining solution for from one to several hours and had taken on a red color, it was used for tissue culture experiments. The specimens were kept for variable periods of time, usually in the icechest, but sometimes in the room, and examined daily during the next two or three or more days. The cells grew out of the piece in a centrifugal direction and gradually extended in the manner described in previous papers.[2] The pieces stained with neutral red either grew about as well as the unstained control pieces, or in some cases they seemed to grow slightly less well. In the amoebocytes, which had emigrated from the piece, the neutral red stain was usually localized in one or several droplets or particles in the interior of the cell. This condition applied to the cells which had spread out in a hyaline condition as well as to the more contracted granular cells. However, in some cases there were still some stained granules visible and occasionally we could observe such a granule, which had

[1] From the Department of Comparative Pathology, Washington University, St. Louis, and from the Marine Biological Laboratory, Woods Hole.

[2] Leo Loeb, Washington University Studies, 1920, VIII., 3. *Science*, 1919, I., 502. *American Journ. Physiol.*, 1921, LVI., 140. *Sicence*, 1922, LVI., 237. Leo Loeb and K. C. Blanchard, *Amer. Journ. Physiol.*, 1922, LX., 277.

assumed the neutral red stain, entering a pseudopod. In a similar way in cells which had grown out into a $n/3$ solution of KCl, and in which circus movements developed in the amœbocytes, one or more stained granules participated in this circus movement in some cases. Even the droplets stained with neutral red seemed occasionally to enter a pseudopod.

Not only the cells which had grown out into Limulus serum and into neutral solutions of sodium chloride contained these droplets or particles of neutral red, but even cells which had grown out into acid ($n/1000$ HCl) and alkaline ($n/200$ NaOH) solutions of sodium chloride showed the typical neutral red droplets.

We have reason for assuming that originally the neutral red entering the cells stained the cell granules and that it was only secondarily deposited in droplets in the amœbocytes. The granule stain evidently had to a great extent disappeared at the time when the slides were examined. However, subsequently even the droplets, especially in the periphery of the field of outgrowth, disappeared in a number of cells in the course of several days. It seemed as if the droplets dispersed gradually into very fine particles and thus were ultimately destroyed. While, as we stated, the cells, as far as their staining with neutral red is concerned, did not at the time of examination show any difference in acid, alkaline and neutral solution, it is possible that later the droplets of neutral red disappeared somewhat more rapidly in the acid than in the alkaline solution; this, however, needs further examination.

However, in many cases the droplets remained unchanged even after the cells had been destroyed and the distribution of these neutral red droplets or particles indicated where cells had been previously.

We made use of this method of staining pieces of amœbocyte tissue with neutral red, in order to study the behavior of cells coming simultaneously from two different pieces of amœbocyte tissue and moving towards each other. For this purpose we placed two pieces, one stained and the other unstained, side by side on a cover glass. Where the two pieces came nearest to each other, the zone of outgrowing cells from both joined and formed a bridge connecting both pieces.

On the whole the tissues derived from the red and from the

unstained piece, each respected the area of the other, but a number of red cells grew into the unstained area in a direction contrary to the centrifugal direction of the majority of unstained cells, and some of the latter grew similarly into the red area. Sometimes the cells moved until they reached the other piece and finding here resistance they turned around and moved back in the direction of their own piece. The cells were able to extend in the strange area and generally underwent the same changes as in their own territory. On the whole, the cells wandering out from the unstained piece remained unstained and did not take up neutral red, which may have become dissolved in the solution surrounding the pieces; but in a few instances, it is probable that such a secondary staining may have occurred in a few cells.

2. In the second method, we allowed tissue to grow out into the surrounding fluid, according to the cover glass tissue culture method, and after a sufficient layer of tissue had thus had a chance to form, we poured off the fluid surrounding the cells and replaced it by an isotonic solution of neutral red in sodium chloride. After this staining solution had acted upon the cells for from one to several minutes, we replaced it by a new solution which was free from stain. We have discussed the results thus obtained in another connection [3] and we shall here merely summarize some of our observations.

Almost instantaneously the neutral red penetrates into the cells and stains the granules red brown. In case the tissue had previously grown out in Limulus serum, the granules of the amœbocytes stain more deeply than if the tissue had grown out in a solution of sodium chloride.

Gradually the granules begin to lose their stain; instead of adhering to the granules, the stain begins to collect in the interior of the cells in the form of droplets or particles which are identical with those described above. If we add a weak acid ($n/1000$ HCl) to the outgrown and previously stained tissue, the granules lose their stain almost immediately. Furthermore, very soon the cells contract in this medium and cease to show amœboid activity. If we replace the acid by a weak alkali, the granule stain usually returns at least in the peripheral cells and a typical

[3] *Am. Journ. Physiol.*, 1924, Vol. 67, 526.

sequence of amœboid activity occurs. A second change of acid and alkali has the same effect as the first change.

This method permits us to observe more completely the effect of the stain on the cells, while in the first method we observed only the later changes.

We see then that acid and alkali have a very marked effect on the decolorization of the granules previously stained with neutral red and these observations suggested to us the experiments reported below on the decolorization of tissue as a whole in acid, alkaline and neutral media; this latter method makes possible a demonstration of the effect of hydrogen ion concentration on the stained tissue without the aid of the microscope.

II. In addition to neutral red we tested the effect of some other stains on amœboid tissue; for this purpose we made use of the first method. We stained the amœboid tissue in toto and used pieces of the stained tissue in tissue culture experiments. As usual the stains were dissolved in a $n/2$ solution of NaCl. On the whole our results with the stains other than neutral red were not very satisfactory.

(*a*) Methylenblue (1:4000), 2 to 2½ hours over tissue in stender dish. Hyaline, as well as granular, cells seem to take on a very slight, diffuse bluish stain; some granules show a more decided bluish coloration. Other cells are hardly stained at all. In some hyaline cells there are some blue droplets, which occasionally show a deeper coloration. There is still amœboid movement noticeable in these cells.

(*b*) Methylviolet (1:1000). In serum less outgrowth than in control; in $n/2$ NaCl very little outgrowth. Granules stain probably very faintly blue.

(*c*) Acriflavine (1:4000). The piece as a whole stains yellow, and the granules also seem to stain yellow.

(*d*) Eosin (1:500). The cells are unstained, although the tissue as a whole has a pink color. The cells grow out into serum as well as into a $n/2$ NaCl solution, but much less than in the control of unstained tissue. The injurious effect of eosin seems to be more marked in the sodium chloride solution than in serum; but even in serum some injury is noticeable. The cells grow out also into acid and alkaline solutions, but show apparently pathological changes.

(*e*) Trypanblue (1:5000). The cells appeared unstained. The outgrowth was similar to control.

(*f*) Janus green (1:4000). Appeared to be relatively toxic. There was apparently a very slight diffuse green stain; neither were granules distinctly stained nor were stained drops or particles visible in the cells. Some amœboid movement was noticeable in this tissue.

III. The Effect of Acid and Alkali on the Decolorization of Amœbocyte Tissue.

Method.—Amœbocyte tissue was prepared in stender dishes in the way described previously.[2] The stain (dissolved in a *n*/2 NaCl solution) was poured over the tissue after the supernatant serum had been poured off. Unless otherwise stated, the stains were used in a dilution of 1:5000.

During the process of staining the tissue was kept in the ice chest. The stained tissue was washed with *n*/2 NaCl solution. Pieces of tissue were then cut out and placed in the small test tubes which contained the solution, whose extractive power it was desired to test. Before comparing the amount of stain given off by the tissue in the various solutions, the pieces of tissue were removed from the tubes and the solutions in the different test tubes brought to the same hydrogen ion concentration and the same volume.

Neutral Red Tissue.—Solutions of *n*/200, *n*/500, *n*/1000 HCl very readily extract the neutral red from the tissue; the extraction usually becomes noticeable to the naked eye within a period of from ten to fifteen minutes or even somewhat earlier. In solutions of *n*/200, *n*/1000 NaOH, not more than a trace of stain is given off. Even after remaining in the alkaline solution for 48 hours in the ice chest very little stain was given off by the tissue. In neutral solutions of *n*/2 NaCl likewise no or very little stain is given off. Neutral red tissue which has been immersed in *n*/1000 and in *n*/500 HCl for 48 hours, and has given off red stain to the surrounding fluid, yields as much color again as it did the first time, if transferred to a fresh acid solution. Other acids, like *n*/1000 benzoic, butyric, lactic acid, extract the stain as well as *n*/1000 HCl.

Eosin Tissue.—Within a few minutes eosin tissue gives off stain readily to the alkaline (*n*/1000 NaOH) solution, a small quantity of stain to the neutral (*n*/2 NaCl) solution and none to the acid (*n*/1000 HCl) solution. This is in accordance with the acid character of the staining radicle in eosin. Eosin differs from neutral red not only in the reversal of the action of acid and alkali, but also in the greater ease with which a neutral sodium chloride solution causes the movement of the stain from the tissue to the solution.

Amœbocyte tissue stained with other stains gives less definite results. Methylviolet is apparently extracted equally by all solutions. Trypanblue does not give off enough stain to make comparisons possible. Acriflavine (1:4000) stains amœbocyte tissue deep yellow; alkaline, acid and neutral solutions seem to extract the stain equally well. Nileblue, on the other hand, bahaves somewhat similarly to neutral red. Acid (*n*/1000 HCl in *n*/2 NaCl) extracts the greatest amount of this stain, *n*/2 NaCl extracts a small amount, but alkali (*n*/1000 NaOH in *n*/2 NaCl) extracts none.

We see then that there is a definite relation between the acid or alkaline character of the dye used, and the character of the solution, which is most effective in extracting the stain from the stained tissue. It is not possible to modify this result if, previous to staining the tissue, we treat it on the following manner: we first allow acid (*n*/1000 HCl) or alkali (*n*/1000 NaOH) in isotonic *n*/2 NaCl solution to act on amœbocyte tissue for a period of three hours. The tissue is then washed with *n*/2 NaCl until the washings are neutral to brom thymol blue, when it is stained with neutral red (1:2000) for two hours, and then again washed until the wash fluid becomes colorless. After such preliminary treatment we found that the tissue previously exposed to alkali stained much lighter with neutral red than tissue exposed to acid or control tissue. Against acid, alkaline and neutral solutions the acid and alkali tissue behaved similarly.

If such acid or alkaline tissue is stained with eosin instead of with neutral red, the tissue behaves exactly like ordinary eosin tissue: the eosin is extracted by alkali, slightly by a neutral solution and not at all by acid.

Effect of Heating Amœbocyte Tissue on the Extraction of Stain.

In various experiments amœbocyte tissue was heated to a temperature varying in different experiments between 60° and 75° for fifteen minutes. This temperature is sufficient to kill the cells; at the same time the heated tissue becomes soft. If such a heated tissue is stained with neutral red and pieces of the stained tissue are extracted with acid, alkali and neutral solutions of sodium chloride in the usual manner, stain is apparently given off in all three solutions. However, if we centrifuge these solutions, we find that in reality only the acid solution was capable of extracting stain from the tissue. In the neutral and alkaline solutions the stain had not actually been extracted, but the soft state of the heated tissue had rendered possible, in the three solutions, the distribution of fine particles of tissue, which were stained and this suspension of stained particles of tissue simulated a real extraction. Amœbocyte tissue killed through heating behaves therefore towards the extraction of neutral red stain like living tissue.

The same results are obtained if we stain heated tissue which had been exposed to acid or alkaline solutions previous to the heating; again, only the acid extracts the stain.

If previously heated amœbocyte tissue is stained with eosin, instead of with neutral red, it is necessary to repeat the process of washing the stained tissue in $n/2$ NaCl about 40–50 times in order to remove a surplus of stain which adheres to the tissue. Tissue thus prepared gives off the stain most readily in an alkaline medium, somewhat less in a neutral, and no stain is given off in an acid medium. The tissue behaves therefore in this respect like living unheated tissue. An exposure of the tissue to acid or alkaline solutions previous to the staining does not alter this result.

Extraction of Stain from Stained Eggs.

Unfertilized eggs of *Asterias* were stained with neutral red, centrifuged and thoroughly washed with a solution of $n/2$ NaCl. Samples, each of two cc., of such an egg suspension were treated with an isotonic acid ($n/1000$ HCl) or isotonic alkaline ($n/1000$ NaOH) solution. The suspension was shaken, centrifuged, the supernatant fluid pipetted into test tubes, and the volume and hydrogen ion concentration were made the same in all the tubes

(corresponding to a $n/1000$ HCl solution). While the tubes, which contained the originally alkaline and neutral solutions, were faintly stained, the acid solution showed the deepest stain. In this case the destruction of a certain number of eggs caused by the procedure used may possibly have complicated the result.

Eggs heated to 50° or 100° for ten minutes and stained with neutral red, gave off the stain even in a neutral solution of sodium chloride. The effect of acid and alkali on the extraction could therefore not be determined in this case.

Corresponding experiments with eggs stained with eosin could not be carried out because unheated starfish eggs do not stain with eosin, and while eggs, heated to 75°, take on a pink stain with eosin, the quantity of stain taken up by the eggs is not sufficiently great to make possible comparisons of the extractive power of acid and alkaline solutions.

Extraction of Stain from Stained Filter Paper.

If filter paper is stained with neutral red, it behaves towards extraction like amœbocyte tissue. For the purpose of extraction the same solutions were used as in the case of amœbocyte tissue. The stain is readily given off in an acid, but not in an alkaline or neutral solution.

Filter paper stained with eosin gives off the stain readily to an alkaline solution, but only a very small amount is extracted by a neutral and none by an acid solution. In other experiments filter paper was stained with trypanblue and subsequently washed in running water for one hour, then shaken with isotonic solutions of $n/1000$, $n/500$ and $n/250$ HCl and NaOH as well as with a solution of $n/2$ NaCl. Small particles of filter paper were suspended in these various fluids. In accordance with the acid character of trypanblue strong alkali extracted the stain, but acid, neutral or weaker alkaline solutions did not.

Discussion

1. The granules of amœbocytes stain readily with neutral red. However, this is only a temporary effect; very soon the granules begin to give off the stain and this loss is almost complete within the course of one or two days. The time at which this change occurs varies somewhat, in some cells it takes place much earlier

than in others. It also depends upon the solution by which these cells are surrounded. In neutral solutions of sodium chloride the large majority of the cells have lost their granule stain within the first 24 hours. But in addition to the granule stain there is a second state in which the stain is found in the cell. It appears in the form of droplets or particles which are usually situated more centrally than the majority of granules, many of which are located in the peripheral part of the cell. Often more than one droplet or particle is present and the size of these droplets varies in different cells. In these droplets or particles the stain is retained much longer than in the granules. They resist also the decolorizing effect of acid more successfully than do the granules. They may persist for some time even after the cells have disintegrated and in such cases they indicate the place where cells have perished. The stain must therefore be fixed much more firmly in these droplets than to the granules. The variations which we find in the number and size of these droplets and particles make it probable that these drops represent cell vacuoles, rather than definite organs, although, if the latter should be the case, it would not alter our conclusions. It is probable that a certain surplus of stain, which cannot be held by the granules, is eliminated into cell vacuoles. In hyaline cells this is the only state in which the stain is found. These droplike formations in which the stain occurs in amœbocytes have some similarity to the droplike formations in which vital stains of an acid character are found in certain kinds of cells. However, such an acid stain, as trypan-blue, does not seem to be taken up to any noticeable extent by amœbocytes.

2. In order to reach the granules the basic neutral red must pass through the outer cell boundary and through the hyaloplasm. In the case of neutral red the cell protoplasm (hyaloplasm) proper is not stained, but certain other basic dyes may perhaps cause a very light diffuse stain. A diffuse stain has been definitely observed in infusoria.

As to the reason why in most cases the protoplasm does not stain under those conditions, we may assume that the affinity of the dye for the granule substance is much greater than for the intergranular protoplasm. In addition it has been assumed that processes of reduction may make the dye invisible in the cell.

As to the relation between the diffuse protoplasmic staining and the granule staining, two views have been expressed. (1) Both the staining of granule and the diffuse staining of protoplasm depend upon the same process, namely, the solubility of the dye in lipoids of the protoplasm as well as of the cell granules (E. Nirenstein [4]); and (2) the staining of granules and protoplasm differ, inasmuch as the diffuse staining of the protoplasm depends upon the lipoid solubility of the stain, while the granule stain is due to a chemical combination between the basic radicle of the dye and an acid constituent of the granule, which latter is presumably not of a lipoid character (W. v. Moellendorff [5]). One argument on which the second view is based consists in the difference in the effects of alkali and acid on the staining of the protoplasm and of the granules. Addition of weak alkali increases the solubility of the dye in lipoids and is therefore believed to favor a diffuse staining of the protoplasm by neutral red. Addition of acid, on the other hand, diminishes the solubility of the dye in lipoids and it is assumed by W. v. Moellendorff that this is the reason why acid prevents the diffuse staining without destroying the staining of the granules. Our experiments prove that the amœbocytes behave differently in this respect: addition of alkali intensifies, while addition of weak acid causes a rapid loss of the granule stain. The protoplasm is not noticeably affected, as far as its staining is concerned, by either alkali or acid. Our observations show therefore that the staining of the granules is affected by acid in the same way as is the protoplasm in certain other cases, and we would therefore conclude that a distinction between the staining of these two cell constituents cannot be based on the argument which we have just cited.

3. According to our observations weak acid decolorizes very rapidly the granules of amœbocytes previously stained by neutral red, while alkali intensifies the staining. As we have seen, the effect of acid and alkali on the staining properties of neutral red has been referred to the influence which acid and alkali exert on the lipoid solubility of neutral red.

On the other hand, Pelet and Andersen [6] have shown that the

[4] E. Nirenstein, *Pflüger's Arch.*, 1920, Bd. 179, 233.

[5] W. v. Moellendorff, *Ergebn. d. Physiol.*, 1920, XVIII., 141.

[6] L. Pelet and N. Andersen, *Zeitschr. f. Kolloidchemie*, 1909, II., 225.

dyeing of wool by acid and basic dyes depends upon the hydrogen ion concentration in the staining solution. They explained this effect as due to the influence of the H and OH ions of the solution on the electrostatic charges of the wool, which latter determine the tendency of the substance to combine with dyes of the opposite charge. They assume this combination to be one of adsorption. Bethe [7] and Rohde [8] apply similar conceptions to the staining of living cells. According to these authors the reaction within the cell determines whether the cells stain with acid or basic stain, and the combination between dye and constituents of the cells has the character of an adsorption. These conceptions are in contrast to those of others who assume that the effect of salts, acids and alkalies on vital staining depends either on the effect of these substances on the permeability of the cell for dyes, or, on their effect on the character of the dye itself. While Bethe believes the combination between constituents of the cell and dye to be one of adsorption, Jacques Loeb [9] showed that proteins combine with acid and basic dyes in a way similar to their combination with ordinary acid and alkali; in both cases the combination is of a stœchiometric chemical nature. In accordance with the amphoteric character of proteid, the latter combines with a basic dye in an alkaline solution and with an acid dye in an acid solution. M. Irwin [11] finds that the entrance of the alkaline dye (cresylblue) into the cell sap of Nitella shows a quantitative relationship to the hydrogen ion concentration of the surrounding fluid, and that these relations can be expressed in an equation characteristic of a monomolecular reaction. She interprets these findings as indicating a chemical combination between the dye and a protein constituent in the cell sap.

Our experiments show that the reaction of the fluid surrounding the stained tissue determines whether the stain remains fixed to the tissue, or, whether it leaves the tissue. We furthermore found that these effects are the same irrespective of the tissue constituent with which the stain had previously combined; it applies in the case of the combination of neutral red with the

[7] A. Bethe, *Biochem. Zeitschr.*, 1922, Bd. 127, 18.

[8] K. Rohde, *Pflüger's Archiv.*, 1917, Bd. 168, 411.

[9] Jacques Loeb, "Proteins and the Theory of Colloidal Behavior," New York, 1922.

[10] Marian Irwin, *Journ. Gen. Physiol.*, 1923, V., 727.

granules as well as in the case of the combination of eosin with other constituents of the tissue. Even tissue killed through previous heating behaves like living tissue as far as the giving off of the stain is concerned, and both behave similar to filter paper which has been stained with acid or basic dyes. On the other hand, a preceding treatment of the tissue with alkali or acid does not alter the effect of the reaction of the surrounding fluid on the decolorization of the tissue.

So far as these experiments show, it seems then that the staining of cell granules, and of other constituents of the cells in amœbocytes, is of a similar character to the staining of cellulose; the surrounding acid or alkali competes with the tissue constituent or cellulose for the dye. The most probable assumption which we can make is that the electrostatic forces of primary or secondary valencies determine the fixation of the dye to the tissue, and that acid and alkali compete with the tissue for the alkaline or acid constituent of the dye. In addition it is very probable that in certain cases acid and alkali influence the result by converting the proteins into salts in which the protein constituent becomes either kation or anion.

While this statement applies as far as the test tube experiments with stained tissue and the microscopic behavior of the cell granules are concerned, it does not apply to the microscopic behavior of the droplike structures in which neutral red is deposited in granular as well as in hyaline cells. These drops seem to a much greater extent to be independent of the reaction of the surrounding medium. We saw that the stain remains concentrated in these drops at a time when in neutral solutions the majority of the granules have already lost their stain. We saw furthermore that these drops remain intact for a relatively long period of time in alkaline as well as in acid solutions, although the latter bring about the almost instantaneous decoloration of the granules. The droplike shape of these structures indicates their liquid character; they represent therefore in all probability solutions of neutral red. It is, however, possible that in addition the dye is deposited also in solid form in certain cases.

These droplike structures may remain preserved at least for sometime, even after the cells have been destroyed, and thus they may indicate the former situation of cells. We must therefore

assume that there must be some factor which prevents these drops from mixing with the surrounding fluid. Two possibilities exist in this respect: either they are surrounded by a protein membrane, or, they consist essentially of lipoid material in which the stain is dissolved.

However that may be, we may conclude that when dissolved in droplike structures the neutral red remains preserved at a time when the neutral red which stains the granules has become dissociated from the latter. We may furthermore conclude that stained granules do not, as has been assumed, represent solutions of neutral red, and that neutral red may therefore be present in the cells in at least two forms, namely, (1) in a chemical combination with the granules, or, (2) in solution in the droplike structures, and that thus the conditions in which the neutral red is deposited in these two cases differ one from another. It may be suggested that the second mode of deposition of neutral red, namely, that in droplet form, is similar to the storage of acid vital stains, like trypanblue and pyrrholblue, which occurs in certain tissues.[11]

Summary.

1. Neutral red, as a representative of the basic dyes, stains the granules of the amœbocytes. The stained granules lose their stain gradually in neutral and almost immediately in acid solutions. Alkaline solutions intensify the staining.

2. In addition to the granules, droplike structures in the amœbocytes are stained with neutral red. The latter are very much more resistant to decoloration than the granules. They do not give off spontaneously the stain as readily as do the granules, nor are they as readily decolorized under the influence of acid as are the latter.

3. The effect of acid and alkali on the neutral red stain in amœbocytes can be demonstrated not only in single cells microscopically, but also in the test tube if we use amœbocyte tissue previously stained with basic or acid stains. Acids cause the giving off of basic dyes, and alkali causes the loss of acid dyes which had previously combined with the tissue. This relation prevails notwithstanding the fact that acid and basic dyes stain

[11] H. M. Evans and W. Schulemann, *D. med. Woch.*, 1914, XL., 1508.

different parts of the tissue. Neither heating nor a preliminary treatment of the tissue with acid and alkali alters this result.

4. We conclude from these experiments that vital stains may be taken up by cells in two forms, (1) through the electrostatic forces of primary or secondary valencies they may be attached to the granules or to the cell protoplasm, or, (2) they may be present in a solution in circumscribed areas of the cell. In the latter form they behave towards environmental factors in a way similar to solutions of the stain. It is possible that also the acid vital dyes exist in the latter form, whenever they are taken into a cell.

THE MANNER OF COPULATION IN A TURBELLARIAN WORM, *PLANARIA MACULATA*.[1]

ROBERT A. BUDINGTON.

During the summer of 1920, while observing rather large numbers of flatworms, *Planaria maculata*, the writer chanced upon a quite ideal opportunity for watching copulation habits in that species. The worms were in a watch-glass which was easily transferred to the stage of a binocular dissecting microscope, and the attitude and relation of the mating worms thus became readily noted. Later they were killed, while still copulating, with a hot corrosive-acetic mixture; and although the worms separated during this treatment, the fixation was rapid enough so that the penes of the killed worms were preserved in very protruded condition.

Referring to the incident in conversation with several zoölogists, it seemed that they had never observed turbellarian copulation, nor did they remember having seen it described. On looking for data regarding it, it has not been possible to find any account of it either in the larger treatises or in more extended special papers on flatworm anatomy and behavior. Curtis, '02, who worked upon the "Life History, Normal Fission, and Reproductive Organs" of this same species for three years, informs me that he did not observe copulation. His studies were so extended, both in numbers used and in period of time, that one is inclined to conclude that either the procedure is not very frequent or that it is of short duration in any given instance.

Since there seems this gap in the recorded descriptions of this worm's total behavior, the following paragraphs are offered as supplementing our knowledge of it, as well as indicating what may be the impregnation process in turbellarians in general; for if, indeed, the process has not been much or at all studied, there may exist even so elementary a question as to whether or not the short copulatory organ is protruded through the atrial pore at all;

[1] From the Department of Zoölogy, Oberlin College, and the Marine Biological Laboratory, Woods Hole.

or whether the spermatozoa may not be deposited in the atrial cavity of each worm and an exchange of them be effected by simple apposition of the pores, as is essentially the case in Annelida, also hermaphrodite.

Contrary to the behavior of oligochæte annelids which adhere in pairs, with anterior ends pointing in opposite directions, two *Planaria maculata* mate with heads in the same direction (Fig. 1).

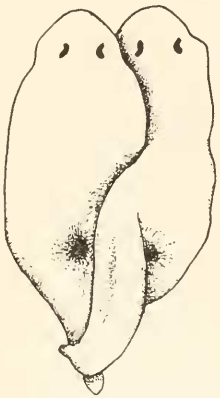

FIG. 1. Attitude assumed by two *Planaria maculata* during copulation.

The anterior ends are maintained side-by-side and flattened on the supporting substance (bottom of watch-glass in this case), oriented alike. About one third of their length posteriorly there begins a rather slight spiral twisting of the flat, oral surfaces of the worms against one another, so that the left ventral side of the right worm of a pair becomes lifted up against the right ventral side of the left worm. This twisting is carried further, posteriorly, so that the tail ends of the worms may even cross one another. At a point on the dorsal surface of each, opposite the external opening (genital pore) of the atrium, there was a marked depression caused by the extension of the penis directly underneath. This indicates that the penis of each worm is drawn into the atrial cavity of the copulating mate by a definite muscular grasp on the part of the walls of the enveloping atrium. This is also suggested by the narrowing of the proximal end of the extended penis; this is evident in Fig. 2. The relation of the two copulants is thus presumably as in Fig. 2; this is purely a diagram, however, and one may not infer that the penes necessarily lie laterally to

one another always, for there may be no constancy in this detail of relations; the laterally side-by-side position, however, would seem better to permit approximation of each copulatory organ to the intra-atrial opening of the uterine duct of its mate.

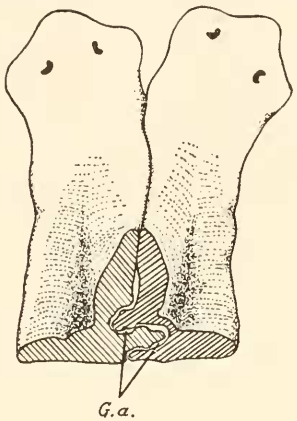

FIG. 2. Diagram of two mating worms cut transversely at level of the genital atria. *G.a.*, Genital atria.

Impregnation in this species is thus mutual and simultaneous between the members of a pair. The period of time over which

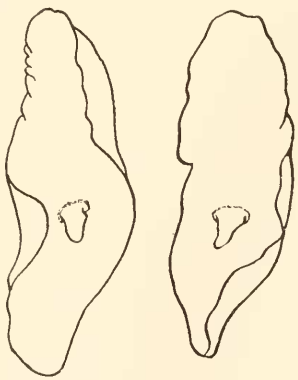

FIG. 3. Outline drawings of two ex-copulant flatworms after fixation. The penes still protrude.

they maintain this relation was not secured as it seemed desirable to fix the worms while copulation was in progress. Fig. 3 shows careful outlines of the two excopulants after fixation, showing relative size of body to copulatory organ, etc.

To determine certain points relating to internal organs, serial sections were made of one of the pair; fixation proved to be satisfactory. As to the normal anatomy of the reproductive organs, the description and figures given by Curtis[1] are wholly adequate and correct. Naturally in the process of copulation the terminal portions of the vasa deferentia become adjusted to the protruded penis; and for the sake of easy comparison, Curtis's

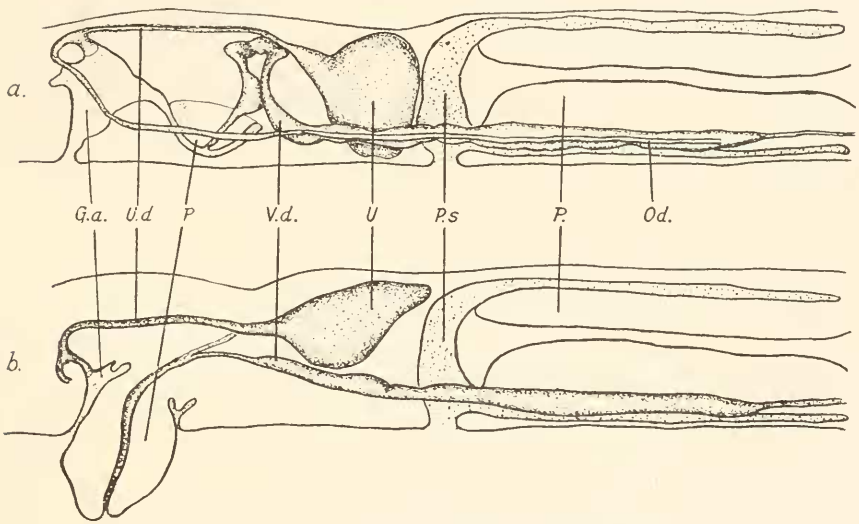

FIG. 4. (*a*) Normal resting position of the reproductive organs of *Planaria maculata*, from lateral aspect. (After Curtis.) *G.a.*, Genital atrium; *Od.*, Oviduct; *Ph.*, Pharynx; *P*, Penis; *U.*, Uterus; *U.d.*, Uterine duct; *V.d.*, Vas deferens. (*b*) Same as (*a*), with organs in position during copulation.

figure of the retracted arrangement of these organs is reproduced beside one drawn from the same aspect with the penis in copulating position. No comment seems necessary by way of interpreting these figures; in mating, the penis is turned posteriorly, extended through the atriopore, and considerably enlarged. This change in position tugs on the vasa deferentia (seminal vesicles) and straightens out the loops which occur in them when at rest.

Concerning the place of deposition and storage of the transferred sperm, a further word may be added. The cavity of the

[1] Curtis, W. C., *Proceedings of the Boston Soc. of Nat. Hist.*, Vol. 30, No. 7, 1902.

atrium is considerably obliterated by the position of the penes; but the remaining space is more or less filled with spermatozoa, as is also the neck of the uterus, although this latter is very narrow and in the sectioned material it shows but a thin trail of sperm. As its wall is heavy with circular muscle, it is probable that it forced its contents along into the uterus while the fixation was still superficial. It also seems likely that few, if any, sperm are retained in the atrium after copulation is over; any such would be expelled through the external pore. The result of copulation will therefore be the reception of spermatozoa into the uterus of each copulant. This sperm mass in the uterus is (at least during copulation as here studied) in the form of a coiled cord or skein-like mass surrounded by mucus.

Gamble states in the section on Turbellaria in the Cambridge Nat. Hist., Vol. II., p. 38, that the copulatory organ when extended is long and narrow enough to reach up into the neck of the uterus; and that the eggs and sperm meet in that cavity. The present observations do not permit a denial of that statement; but the size of the organ as against that of the uterus duct as seen in Fig. 5 seems to make such an insertion, in *Planaria* at least, very improbable.

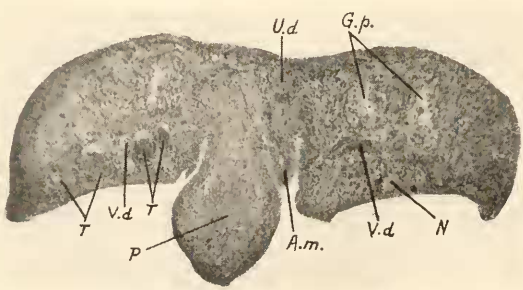

FIG. 5. Transverse section of *Planaria* at level of atrium, during copulation. *A.m.*, Atrial margin; *G.p.*, Gut pouches; *N.*, Nerve; *P.*, Penis; *T.*, Testes; *Ud.*, Uterine duct; *V.d.*, Vas deferens.

Again, although it is well known that in many forms the eggs are passed up into the uterus and meet the sperm there, in the present instance the uterus, as shown in sections, contains only sperm embedded in a mucus mass. The statement is often made that the sperm cells are aggregated into spermatophores; in a

study of a single section this does seem to be the case, but further examination shows the spermatozoa to be in a long skein, much coiled, and surrounded by mucus. For a time at least the uterus may function merely as a sperm receptacle.

Summary.

1. *Planaria maculata*, and probably many other triclad Turbellaria, mate with both copulants oriented in the same manner.

2. Impregnation is mutual, and simultaneous.

3. During copulation the vasa deferentia are much distended by their sperm contents, while the uterus may act temporarily as a sperm receptacle only.

THE INFLUENCE OF HYDROGEN ION CONCENTRATION ON UNFERTILIZED *ARBACIA, ASTERIAS* AND *CHÆTOPTERUS* EGGS.

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In a previous paper the writers briefly reported experiments on the effects of increased H-ion concentration in sea water on the fertilization and development of the normally fertilized eggs of the star fish (*Asterias forbesii*) and sea urchin (*Arbacia punctulata*) (1). This work has since been repeated and extended in several directions and more complete reports on these subjects are now ready for publication. The experiments to be reported in this paper deal with effects of acid and alkaline sea water on the rate of ageing of unfertilized *Arbacia, Asterias* and *Chætopterus* eggs, and with the artificial activation of *Chætopterus* eggs by acid sea water. These experiments, beside affording a necessary basis for further studies of the influence of variations in H-ion concentration on the fertilization and developmental processes, furnish important information on the relation of the physiological activity of the egg cell to its environment.

Preparation of Acid and Alkaline Sea Water.

Certain of our experiments, which will be described in a subsequent paper, have shown that CO_2 exerts a profound effect, distinguishable from the effects of H ions, on many of the physiological processes of marine eggs. Since sea water naturally contains a considerable quantity of combined carbonic acid, it is necessary in all experiments designed to observe the effects of H ions *per se* to work with sea water from which the CO_2 has been removed. The CO_2-free sea water used in these experiments was prepared as follows: To each liter of fresh sea water was added 5 cc. of $N/2$ HCl and 5 cc. of $N/10$ NaH_2PO_4; this mixture was aërated with a water vacuum pump over night. No pro-

vision was made for excluding the CO_2 of the atmosphere because the concentration in equilibrium with the atmosphere after aëration is, so far as our experiments are concerned, negligibly small. $N/40$ NaOH was used for restoring this acid sea water to the desired H-ion concentration, using colorimetric standards with a salt content equivalent to that of sea water prepared as recommended by Clark (2) and McClendon (3). NaH_2PO_4 was added to the acidified sea water to give it greater buffering capacity in the neighborhood of neutrality, a rôle normally played by $NaHCO_3$. A sufficient quantity of acid sea water was made fresh for each day's use, and the individual solutions were prepared immediately before using.

CO_2-free sea water prepared in this manner was used between pH 4.5 and 8.0. In solutions more alkaline than pH 8.0 basic phosphates are thrown out, so solutions more alkaline than sea water were prepared by adding $N/10$ NaOH to natural sea water. In this case the carbonates do not interfere because the CO_2 tension is negligibly small. At pH 10.2 $Mg(OH)_2$ begins to precipitate; the amount of alkali required to complete this precipitation is many times the amount required to bring sea water from its normal reaction to 10.2. If the precipitation of $Mg(OH)_2$ were completed, the resulting solution would be physiologically unbalanced. It is, therefore, impossible to go beyond this point on the alkaline side. Consequently our observations are limited to the range pH 4.5 to 10.2, which embraces all the physiological variations with which we are concerned.

The addition of HCl and NaOH dilutes the salt content of the sea water slightly but we have no reason to believe that this slight degree of hypotonicity has introduced any serious complication into our results. Nearly all experiments reported in this paper were performed at approximately 20° C. Early in the season it was necessary to bring the sea water to this temperature, but later it was easily maintained with a slight regulation of the temperature of the laboratory.

The Viability of *Arbacia* and *Asterias* Eggs.

Asterias eggs were left in sea water until the maturation process was practically completed, during which time they were

washed and gently agitated several times. *Arbacia* eggs were washed with sea water twice after removal from the ovaries and then used at once. The eggs were gathered into a concentrated suspension by gently centrifuging and a few drops of this suspension added to 200 cc. of the pH solutions contained in finger bowls. A small quantity of eggs was used so that there would be little crowding when the eggs settled to the bottoms of the bowls. During the exposure they were frequently agitated. At various intervals samples were transferred to sea water and inseminated with fresh sperm. The samples were examined after the eggs had had time to divide, noting both the number which fertilized, as shown by the presence of a fertilization membrane, and the number which had divided.

The results of several experiments with each species have shown that, as might be expected, there is some variation in the behavior of eggs from different individuals. The extremely artificial method by which the eggs are obtained—that is the excision of the ovaries and the consequent forced shedding—is not a method which could be expected to give eggs of uniform physiological quality from several individuals. But differences in the actual time of survival of eggs from different individuals, though important in some respects, do not materially alter the relative time of survival at different H-ion concentrations. Since the actual time of survival in any one experiment has no particular significance, it has been thought best to omit the extensive tabular data and to express the results in diagrammatic form with such quantitative expression as can reasonably be implied.

The relations between viability and H-ion concentration of *Arbacia* and *Asterias* eggs are given in Figs. 1 and 2. In these figures each contour line represents the proportion of eggs surviving at a definite interval after transfer to the pH solutions, the time of testing being marked on each curve. The data are based on the average results obtained from several experiments. The dotted curves marked "cytolyzed" show the proportion of cytolyzed eggs at the conclusion of the experiments. The full ordinate in these and all following diagrams indicates the H-ion concentration of sea water, *i.e.*, pH 8.15.

It might be supposed that marine eggs would retain for the

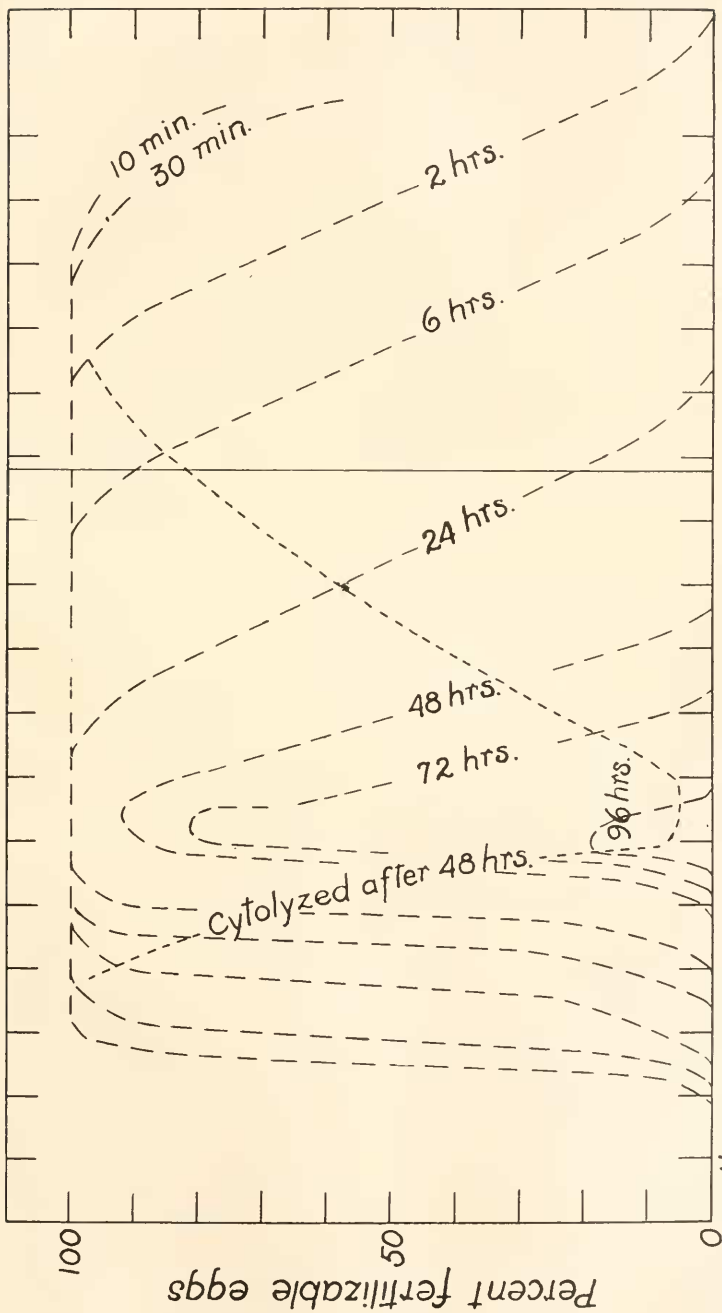

FIG. 1. The proportion of *Arbacia* eggs surviving after the stated exposures to acid and alkaline sea water.

longest time their capacity to fertilize (or to develop) in their normal habitat, sea water, the H-ion concentration of which rarely varies beyond pH 7.8 to 8.4. Contrarily, both *Arbacia* and *Asterias* eggs retain their viability longest, and are least susceptible to cytolysis, in quite acid solutions; the optimum reaction for *Asterias* lies between pH 6.2 and 6.6, and for *Arbacia,* 5.8 to 6.0. The eggs of both species die with great rapidity in very alkaline (pH 10.0) and very acid (pH 4.5) solutions; the rate of injury appears to decrease uniformly as the optimum is approached, particularly from the alkaline side. There is no disproportionate alteration in the rate of death around the pH of sea water. It will be noted that the optimum does not occur midway between the extremes but nearer the acid limits. That is, with respect to the optimum, the eggs of both species can tolerate a larger increase in alkalinity than in acidity. The *Arbacia* egg retains its fertilizing capacity much longer at all H-ion concentrations than does the *Asterias* egg.

It is well known that batches of *Asterias* eggs are frequently encountered in which all the eggs will not maturate or, even when maturated, will not fertilize. Ralph Lillie (4) has shown that the general physiological condition of such eggs could be greatly improved by treatment with ether, as shown by an increase in the number of eggs which both fertilize and divide. Similarly we find that after a short exposure to slightly alkaline sea water (pH 8.2 to 9.4), the proportion of fertilized eggs in these refractive lots is increased. This is expressed in Fig. 2 by the slightly higher incidence of fertilization on the alkaline side of sea water. This effect is clearly a more or less permanent alteration of the egg, since the eggs were transferred from the alkaline solution to normal sea water before insemination. It has sometimes been observed that the proportion of fertilizable eggs is decreased by a short exposure (3 to 5 minutes) to pH 10.0–10.2, and subsequently increased by longer exposures, short of permanent injury. No reason can be given for this apparent transient injury.

Goldfarb (5) has made careful studies of the consequences of ageing in sea water of three species of sea urchin eggs. *Arbacia, Hipponoë* and *Toxopneustes.* He finds that with increased ageing there is an increased tendency for agglutination, fusion of

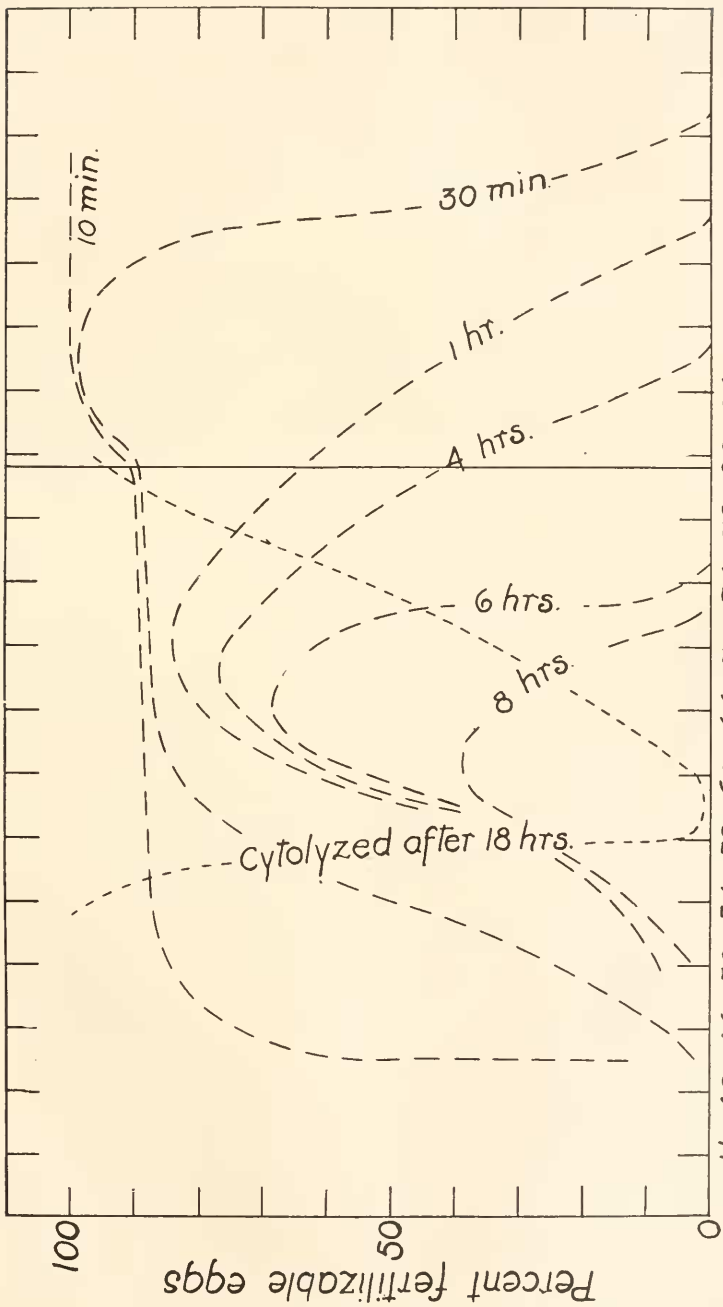

FIG. 2. The proportion of *Asterias* eggs surviving after the stated exposures to acid and alkaline sea water.

eggs and blastulæ, irregular cleavage as manifested in change of size and shape of the blastomeres, retardation in the rate of cleavage, and in extreme stages a total loss of the capacity to cleave. There is also an increase in volume and a loss of the jelly normally surrounding the fresh egg; in *Toxopneustes* and *Hipponoë* there is an initial acceleration in the rate of membrane formation, and in all three species a subsequent retardation of this rate and ultimately a complete loss of the capacity to lift a membrane. Goldfarb attributed the changes accompanying ageing principally to changes in the cortical layer, which, he states, are in turn referable to changed metabolism.

Our results confirm Goldfarb's findings in respect to the loss in *Arbacia* of the capacity to divide. This is equally true at all H-ion concentrations. In *Asterias,* however, on the alkaline side of the optimum the capacity to divide is lost before the capacity for membrane formation. But on the acid side of the optimum (pH 6.2 to 6.5) practically all eggs which lift membranes develop through the first few cleavages.

In both species the tendency for polyspermy increases in proportion to the physiological, rather than the temporal, age of the eggs. Consequently, the incidence of polyspermy is decreased at the optimum to about the same extent to which the viability of the eggs is increased. It is difficult to distinguish polyspermic from abnormally dividing eggs without cytological examination, and therefore it is deemed inadvisable to draw conclusions from our data concerning the tendency for polyspermy after ageing at various H-ion concentrations. It may be said, however, that among those eggs which are aged from pH 6.2 to 6.5 there is a decidedly lower incidence of both definite polyspermy and irregular division, as contrasted with eggs which are aged in more alkaline solutions. The former, if they divide at all, tend to divide regularly through at least two or three cleavages.

The ageing of *Arbacia* and *Asterias* eggs in sea water is accompanied by a slight increase in volume and fluidity. The nucleus which is difficultly discernible in the fresh mature egg, appears in the stale egg as a distinctly defined, hyaline vesicle near the center of the egg. Later, when the egg loses its fertilizing capacity, the cytoplasm becomes distinctly granular and opaque and

the even contour of the egg is lost. As Chambers (6) has shown by microdissection, the granules in the dead egg are disintegrative products and not comparable to the granules in the living egg. They are no longer glutinous or adhesive; the egg has entirely lost its original homogeneity and is held together only by the investing vitelline membrane. Gradually this disintegrative mass imbibes water and swells within the vitelline membrane, becoming a more or less vacuolated liquid mass. Ultimately, the membrane breaks and the contents are dissipated in the sea water.

When *Asterias* eggs are allowed to age in acid and alkaline sea water, the transformation of the nucleus and the subsequent granulation of the cytoplasm occurs most rapidly in solutions more alkaline than sea water, and at about the same rate from pH 8.0 to 5.4. At acidities greater than pH 5.4 the nuclear transformation is perceptibly retarded and the cytoplasm acquires a granular appearance which differs from that of eggs aged in more alkaline solution principally by a diminished degree of discoloration.

From pH 5.4 to 6.2 many eggs are observed which contain, instead of a single vesicular nucleus, two, three or more smaller contiguous vesicles. Such eggs are observed much less frequently in solutions more alkaline than the optimum, pH 6.2. These polyvesiculated eggs will, when returned to sea water and inseminated, lift normal, turgid fertilization membranes in 3 to 5 minutes, and will usually cleave simultaneously into several blastomeres. If not inseminated when returned to sea water, a very small per cent. of the polyvesiculated eggs will fragment once or twice, the vesicles apparently being distributed among the fragments. Although the process of migration of these vesicles into the fragments prior to cleaving was not observed, they appear to be causally related to the process of fragmentation. Fertilization membranes are not formed spontaneously on the polyvesiculated eggs either in the acid by exposures of two to three hours, or when returned to sea water; though a few eggs will form fertilization membranes if left in the acid solutions for considerably longer periods, 4 to 6 hours. The fragments are held together by a delicate membrane bridging the furrows; this may be the vitelline membrane of the unfertilized egg.

The nucleus of the *Arbacia* egg acquires a vesicular appearance

on ageing much the same as that of the *Asterias* egg. As in the latter case this change occurs in about the same time at all H-ion concentrations from pH 5.0 to 10.0. At acidities greater than pH 5.0 there is an increased incidence of eggs in which this nuclear change does not take place. We have not observed the appearance of several vesicles in the *Arbacia* egg at any H-ion concentration, but our observations would not preclude their existence.

In solutions more alkaline than pH 9.0 there is a tendency for membranes to lift spontaneously on both *Asterias* and *Arbacia* eggs. Prior to membrane formation the cortex of the egg undergoes peripheral disintegration with formation of droplets. Spontaneous membrane formation decreases with increasing acidity; below pH 7.0 it is rarely observed except when induced in *Asterias* eggs by long exposures to pH 5.4–6.2, as mentioned above.

Changes in Physical Properties.

The appearance of eggs which have been exposed for a short time to extremely acid or alkaline sea water is markedly different. Alkali treated eggs present a smooth, almost glassy surface, while acid treated eggs are dull and appear to have a finely granulated surface. The slightest amount of manipulation indicates that the alkali treated eggs are soft and more liquid than normal, while eggs treated with acid are more solid. Dr. Chambers has kindly examined the effects of acid and alkali on these eggs by means of microdissection. He finds that in acid sea water the thin, delicate vitelline membrane which normally surrounds the unfertilized *Arbacia* and *Asterias* egg is toughened so that it is difficult to tear. This toughened membrane makes it difficult to ascertain mechanically what influence the acid solution may have on the consistency of the egg surface itself, but with non-injurious exposures acid seems to produce no very profound change in the consistency of the egg cortex. Longer exposures lead to a gradual setting or gelation of the cortex (and possibly the egg as a whole), finally accompanied by the loss of its normal transparency.

In alkali both *Asterias* and *Arbacia* eggs are rendered extremely plastic, soft and liquid by short treatment. This can be shown by shaking the eggs for a uniform time in solutions of increasing alkalinity. Thus when *Asterias* eggs are vigorously shaken in sea

water of pH 5.0 to 10.2 after an exposure of 10 minutes, very little cytolysis or fragmentation occurs between pH 5.0 and 9.0. At pH 9.3 there is a slight amount of fragmentation and a slightly increased number of cytolyzed eggs. A large number of the eggs are distorted from their normally spherical shape, showing that they have softened. At pH 9.6 a few eggs are broken into smaller fragments and the number of intact but cytolyzed eggs is diminished as compared with 9.3. At pH 9.9 the membrane and egg cortex are abruptly destabilized and all the eggs are readily broken into small, spherical and extremely stable fragments.

Similarly the *Arbacia* egg is comparatively resistant to moderate shaking between pH 5.8 and 9.3. At about pH 9.6 there is a marked increase in the tendency to cytolyze. This egg does not fragment as does the *Asterias* egg, presumably because of its inability to form new surface films readily, but appears to cytolyze rather slowly after rupture at some one point. From pH 9.6 to 10.2 the shaken suspensions are filled with ghosts and partially cytolyzed eggs.

The Maturation of *Asterias* Eggs.

The maturation of *Asterias* eggs is normally initiated as soon as they are removed from the ovaries and come in contact with sea water. The initiating factor or factors are not known. Loeb (7) has shown, however, that the addition of acid to sea water inhibits, and the addition of alkali favors the maturation process. When slowed below a critical velocity the maturation process stops and the eggs remain permanently immature.

In view of the possible rôle of H- or OH-ions in initiating maturation, an examination was made of the effects of increasing acidity on the incidence of permanently immature eggs. The eggs were introduced into the pH solutions without contact with sea water by dipping small pieces of fresh ovary into the pH solutions. After 45 minutes or an hour counts were made of the mature and immature eggs, discriminating by the dissolution of the wall of the germinal vesicle. A summary of experiments of this nature is given in Table I.

TABLE I.

NINE EXPERIMENTS ON THE INFLUENCE OF H-ION CONCENTRATION ON MATURATION OF *Asterias* EGGS.

Per Cent. Permanently Immature.

pH									
6.0	6.2	6.4	6.6	6.8	6.9	7.0	7.2	7.4	8.15
	91	72	58	32	28	45	30	6	0
	88	20	44	40	23	40	12	4	0
99	90	82	43	26	8	27	5	1	0
	58	34	22	16	8	18	10	4	0
	76	80	68	63	48	50	35	31	0
	93	76	69	46	60	64	25	10	1
95	88	71	61	39	36	26	14	5	8
93	86	68	69	57	33	16	17	5	1
	76	20	28	33	39	21	17	4	5

Maturation is practically inhibited at pH 6.0, a point at which normally fertilized *Asterias* eggs will grow quite normally, and approximately the point at which the unfertilized egg retains its viability for the longest time. It should be noted in Table I. that the incidence of maturated eggs does not always increase uniformly with increasing alkalinity, but that in some experiments the proportion of immature eggs falls to a low value on the acid side of neutrality, rises noticeably at pH 7.0 (or 6.9) and then falls to zero at 8.15. This irregularity in the influence of H-ion concentration on the maturation process is not affected by washing several times in the respective pH solutions. It appears, therefore, to be attributable to alterations in the egg cortex rather than to the activity of some substance in the supernatant fluid.

THE INFLUENCE OF H–ION CONCENTRATION ON *Chætopterus* EGGS.

The *Chætopterus* egg differs from echinoderm eggs in that it is activated by sea water to which HCl has been added, as Loeb showed many years ago. This activation, though not qualitatively nor quantitatively sufficient to produce normal larvæ, makes it necessary to consider separately the consequences of exposing the unfertilized egg to acid solutions, and the effects of such exposures on subsequent fertilizability.

Like the egg of *Asterias,* the *Chætopterus* egg is shed immature; though the germinal vesicle breaks down when the egg is placed in

sea water, maturation proceeds only as far as the formation of the first maturation spindle. Unless the egg is fertilized or artificially stimulated it remains in the metaphase of the first maturation division. After fertilization the maturation divisions are completed and the polar bodies are formed at the animal pole. Mead (8) first called attention to the fact that a small amount of KCl added to sea water causes these eggs to complete the maturation process with polar body extrusion, an event in this case signifying initiation of development. Three years later Loeb (9), having succeeded in obtaining parthenogenetic development of echinoderm eggs by the use of hypertonic sea water, tried similar procedures on *Chætopterus* eggs. He also examined the influence of KCl and concluded that activation in this case was not a consequence of increase in osmotic pressure, but a specific effect of K-ions. In a careful description of the parthenogenetic development of this egg, he pointed out that after treatment with KCl, development and differentiation appear to proceed without cell cleavage (an observation subsequently confirmed by F. Lillie, 10), and that activation of the egg is accompanied by marked ameboid movements of the protoplasm. He did not succeed at this time in obtaining development with cleavage, though later in collaboration with Wasteneys he obtained cleavage by the combined use of $SrCl_2$ and ox serum. Loeb also decribed a marked tendency in *Chætopterus* eggs activated by KCl to adhere to each other; as a result of this adhesion, larvæ might be formed consisting of several swimmers partially fused, or in some instances, of giant swimmers resulting from the complete fusion of several eggs.

Loeb also obtained ciliated, unsegmented larvæ by treating *Chætopterus* eggs with 100 cc. sea water + 2 cc. $N/10$ KOH, and with 100 cc. sea water + 2 cc. $N/10$ HCl. He mentions the fact of activation by HCl as striking since he had failed to get activation of echinoderm eggs by similar treatment. (It was not until five years later that he tried the fatty acids with signal success.) Allyn (11) has recently examined the action of several acids on *Chætopterus* eggs. She failed to get segmentation and concluded that acids are less effective than KCl.

In our experiments with *Chætopterus* we have observed the

effect of acid and alkaline sea water both in respect to activation and to the subsequent fertilizability after varying exposures. The worms were removed from their tubes as soon as they were brought into the laboratory, and the males and females were placed in separate dishes with running sea water. Before use the females were rinsed well with tap water, then with sea water, and placed in about 50 cc. of sea water in a finger bowl. The egg sacks were cut and the ovaries removed and gently teased apart. After about 15 minutes when all the ripe eggs were shed, the tissue fragments were picked out, the egg suspension filtered through cheese cloth and the eggs concentrated by centrifuging. Because of the small quantity of eggs available, it was necessary to reduce the volume of the pH solutions to 50 cc. Equal quantities of maturated eggs were added to each of the pH solutions; at various intervals portions of these eggs were transferred to two dishes of sea water, only one of which was inseminated. Fresh sperm from one male were obtained when desired by cutting a new sperm sack and allowing the escaping sperm to accumulate in a small quantity of sea water.

The activation of *Chætopterus* eggs by H-ions is illustrated in Fig. 3 by the dotted line. The activation is most intense at pH 5.8 and diminishes rapidly on either side of this point, practically disappearing at 5.0 and 6.6. If the eggs are left at pH 5.8 polar bodies are extruded in 30 to 50 minutes, and about 50 per cent. or more will at the end of two or three hours show marked ameboid movement and extensive fragmentation by budding. The egg flows spontaneously into several unsymmetrical pseudopodia and these in turn develop smaller extrusions, many of which bud off into small spherical fragments. Nuclear division apparently does not always precede this fragmentation, which seems to be largely a result of the ameboid activity of the cortex. If, however, the eggs are exposed for one or two hours to the acid solution and then returned to sea water, 50 per cent. or more will undergo one or two segmentations which more or less simulate the divisions of the normally fertilized egg. They do not develop beyond the two- or four-cell stage, however, and in the majority of instances the cleavages are irregular and the blastomeres tend to separate. If the eggs are returned to sea water after shorter ex-

posures a still smaller proportion of them show signs of activation. A 30-minute exposure will produce polar body formation but only a few eggs will fragment. A 10-minute exposure is insufficient to activate at any H-ion concentration; attention is called to this point because this exposure produces changes in the egg which prevent fertilization by sperm.

There is also a slow activation in alkaline sea water, beginning about pH 9.6 and increasing to 10.2. The activation is apparently not so intense as at pH 5.8 since an exposure of 4 to 5 hours is required to induce segmentation.

It should be emphasized that the H-ion activation of *Chætopterus* eggs is not strictly comparable to the activation of echinoderm eggs by the fatty acids. Loeb showed that in *Arbacia* the strong acids were practically incapable of activating; the fatty acids are efficient by virtue of their penetrating power. Loeb obtained slight activation of *Asterias* eggs by treatment with sea water acidified with HCl (7), but we have obtained no activation of either *Asterias* or *Arbacia* eggs by CO_2-free sea water as acid as pH 4.5. It is probable that the activation obtained by Loeb was due to free CO_2, which Delage has shown to be an excellent activating agent (12).

If *Chætopterus* eggs which have been exposed to the pH solutions for 10 minutes are returned to sea water and inseminated, those from solutions in the neighborhood of pH 5.8 will not fertilize; they remain inert when the eggs taken from the solutions at pH 4.6 or 7.6 have fertilized and have undergone two or 3 normal cleavages. A 10-minute exposure to these solutions, though insufficient to activate, apparently produces some block to fertilization which is not produced by equal exposures to pH 4.6 or 7.6, or to the alkaline solutions which also activate. (The solid line in Fig. 3 shows the proportion of eggs which fertilized in sea water after an exposure of 10 minutes to the pH solutions.) But if the eggs are left in the pH solutions for 30 minutes or longer before being transferred to sea water and inseminated, they slowly recover their fertilizability, in as much as the addition of sperm causes them to segment normally to the 16- or 32-cell stage, and to develop into swimming larvæ of more or less normal appearance. The longer the exposure to the pH solutions, the greater the pro-

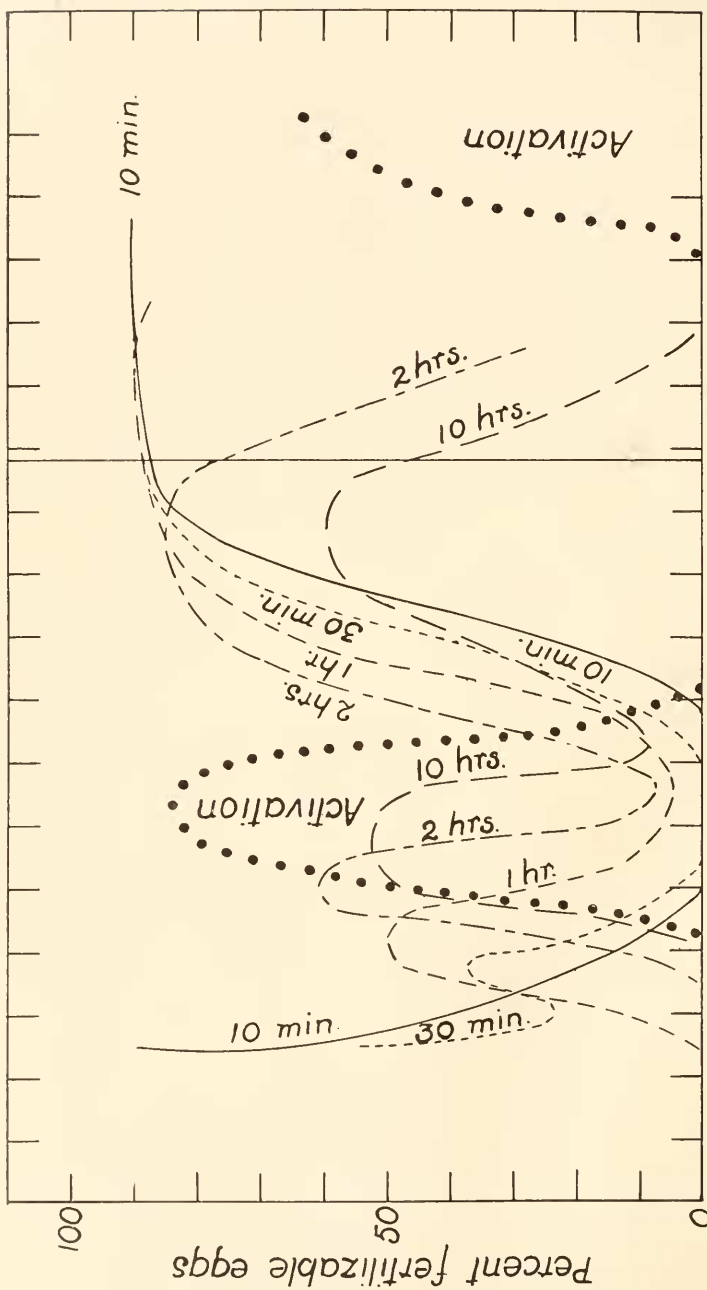

FIG. 3. The heavily dotted lines show the regions of artificial activation of *Chaetopterus* eggs by acid and alkaline sea water. The solid and the dashed lines show the proportion of eggs which fertilize when returned to sea water and inseminated after the stated exposures to the pH solutions. A block to fertilization is created by short exposures to those solutions having the greatest activating power; when the eggs are left in these solutions this blockgradually disappears.

portion of eggs which recover their fertilizability. (The dashed contour lines in Fig. 3 show the general course of this recovery process. The figures written beside each curve show the duration of the exposure to the pH solutions.) The block to fertilization produced by short exposures to the solutions around pH 5.8 disappears only when the eggs are left in the acid solutions. If returned to sea water after a short exposure (*i.e.*, 10 to 30 minutes) the eggs remain permanently (4 to 6 hours) unfertilizable.

Dr. Chambers has examined these eggs by microdissection and finds that when treated for 5 minutes at pH 5.8 and then placed in sea water, the membrane which envelops both the fertilized and unfertilized egg is very much thickened and toughened. Longer exposure to pH 5.8 tends to soften this membrane so that when returned to sea water it is thin, delicate and easily torn. Though the conclusion is by no means substantiated, it is possible that this initial toughening of the membrane with subsequent softening on longer exposure accounts for the inability of sperm to react with short exposure as compared to long exposure eggs.

That the sperm gain access to the egg after the block has worn off is shown by the fact that without sperm they undergo at the most two or three cleavages which are decidedly late and irregular, while with sperm they develop with much more normal velocity and with a quality that is so nearly normal that in many instances they cannot be distinguished from normally fertilized eggs. Many of them, moreover, develop into rough swimmers. There is a marked tendency for the blastomeres to fuse in the later stages with the production of syncytia; and for the separate blastulæ to fuse, 4 or 5 forming one large, apparently homogeneous larva, or for several to adhere together forming irregular chains. This tendency for fusion, like the ameboid movements which accompany activation and normal division, is clearly a consequence of some lability of the cortex. Fusion appears to be more marked among those eggs exposed to solutions on the acid side of pH 5.8 than on the alkaline side.

If we neglect the temporarily irreversible block created at pH 5.8, the optimum reaction for the retention of viability will probably be somewhere in this neighborhood, *i.e.*, pH 5.8 to 6.0. It is certain that this optimum is considerably on the acid side of sea

water, as is the case in *Arbacia* and *Asterias,* and comparatively close to the limits of acid tolerance.

The scarcity of material made it impossible to examine more closely the influence of reaction on the physical properties of the *Chætopterus* egg. It may be stated, however, that unlike *Asterias* and *Arbacia,* the *Chætopterus* egg is distinctly liquified rather than solidified at pH 4.6 to 5.0. This liquefaction is so marked that after an exposure of one or two hours the eggs are extremely fluid and will flow into thin pencils when the containing vessel is tilted or jarred. A similar liquefaction takes place from pH 9.0 to 10.0.

There is no doubt that physical changes in the nature of coagulation in acid and dispersion in alkali characterize the limits of physiological tolerance in *Arbacia* and *Asterias* eggs. But that coagulation in acid is not the invariable rule is evident from the liquefaction which occurs in *Chætopterus* eggs. It is reasonable to suppose that the specific composition of the cortex (and perhaps of the vitelline membrane as well) determines both the direction and degree of the physical changes at various reactions. The cytoplasm of all the eggs examined here is distinctly liquid (13) and it may be that the liquefaction or gelation observed is more a consequence of changes in the vitelline membrane and cortex than in the cytoplasm proper.

The general nature of the changes in physical properties and the changes accompanying ageing at various H-ion concentrations suggests that the prolongation of the life of these eggs in acid solution is a consequence of reduced metabolism. Increasing acidity up to a certain point leads, perhaps by an internal action or by a reversible alteration in the cortex which decreases the facility of interchange, to decreased metabolic activity; excessive acidity on the other hand produces irreversible injuries in the egg; where reduced metabolism and acid injury strike a reversible mean, the egg retains its viability for the longest time. The agreement between the pH at which maturation of *Asterias* eggs is completely inhibited and the pH of maximum viability conforms with this suggestion. The H-ion concentration of maximum viability may have some significance in relation to ovarian life, for in the ovary the egg is subjected to a greater CO_2 tension and H-ion concentration than that of sea water. But the cœlomic

fluids of *Asterias* and *Arbacia* are approximately neutral and it is unlikely that at any time the egg would naturally be subjected to an acidity so great as pH 6.0.

Summary.

A method is described for preparation of CO_2-free sea water of H-ion concentrations from pH 4.5 to 10.2.

The eggs of *Asterias, Arbacia* and *Chætopterus* retain for the longest time their capacity to fertilize and to divide at about pH 6.0.

Approximately this same H-ion concentration is required to completely repress the maturation process in *Asterias* eggs.

Chætopterus eggs are activated by exposures of 30 minutes or more to solutions of pH 5.0 to 6.6. If left in these solutions they show marked ameboid movements and fragmentation, but do not divide. If returned to sea water half or more of the eggs will undergo one or two abortive divisions. The activating effect of the acid sea water is most intense at about pH 5.8 to 6.0.

An exposure of 5 or 10 minutes to solutions of pH 5.0 to 6.6 (which is insufficient to activate) creates a block to fertilization which is permanent if the eggs are returned to sea water. If left from 30 minutes to several hours in the acid sea water, these eggs gradually recover their fertilizability, and when inseminated develop almost normally.

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THE INFLUENCE OF HYDROGEN ION CONCENTRATION ON THE DEVELOPMENT OF NORMALLY FERTILIZED *ARBACIA* AND *ASTERIAS* EGGS.

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The belief that cessation and initiation of development in the marine egg depended in some manner on the ionic equilibria of sea water led Loeb (1) to examine the influence of changes in the concentration of H- and OH-ions on the development of normally fertilized eggs. He found that the development of the eggs of *Arbacia* is retarded and finally prevented if increasing quantities of acid are added to sea water, and that the development to the pluteus stage is accelerated in alkaline sea water. The latter fact was indicated by the advanced size and development of the plutei formed from the treated eggs as compared with controls. On subsequent investigation he concluded that alkali does not accelerate the early cleavage rate, but only the later development from the blastula to the pluteus. The addition of excessive quantities of alkali had an injurious effect. The maximum stimulation was observed when 1.75 cc. *N*/10 NaOH were added to 100 cc. sea water. He attempted to raise the newly fertilized eggs of *Strongylocentrotus* in a neutral Ringer's solution without success, but found that with the addition of a small quantity of KOH, or better $NaHCO_3$, good larvæ might be obtained. He concluded that a neutral or faintly alkaline solution is necessary for normal development (2). This conclusion was reached from other points of view by Herbst (3) and Peter (4).

Moore, Roaf and Whitley (5) performed similar experiments with the eggs of *Echinus esculentus;* the addition of small amounts

of alkalies or alkaline salts, such as Na_2HPO_4, to sea water in which the eggs were growing caused an increase in the rate of growth in the early as well as the late stages, but larger amounts led to abnormal division. They pointed out that in some eggs in quite alkaline solutions nuclear division occurred without cytoplasmic division, so that the blastomeres became multi-nucleated. Still larger amounts of alkali inhibited both nuclear and cytoplasmic division. On the other hand, the smallest amount of acid had only an inhibitive action. There was no tendency for nuclear division without cytoplasmic division; and with comparatively small amounts of acid cell division was completely prevented. They concluded that the extreme limits of reaction at which cell division is possible lie very close together, and they pointed out that the phosphates and carbonates in sea water have a "steadying action" against fluctuations in the concentrations of H- and OH-ions which must be advantageous to cell growth. Subsequently, Whitley (6) found that small quantities of acid and alkali were very injurious to the developing eggs of the plaice, *Pleuronectes platessa*. No accelerating effect was observed in alkaline sea water, but Whitley concluded that a disturbance of the equilibrium towards the acid side is much more fatal than the opposite. There appeared to be an increase in resistance to unfavorable reactions developed in proportion to the age of the larvæ.

Glaser (7) repeated Loeb's experiments with *Arbacia* in another connection and concluded that accelerated development in alkaline sea water is limited to the development from the blastula to the pluteus, and that the early rate of cleavage is not accelerated, and may even be suppressed. Glaser noted the time required for the successive cleavage planes to appear in the majority of eggs in the cultures. By this method a small change in velocity of development would be difficult to detect, though it would become manifest if continued to the later stages where its results would of course be magnified.

Although it had another objective, the excellent work of Medes (8) on the causes of variation in the larvæ of *Arbacia* is of interest in this connection. Medes made careful comparative measurements on the skeletons of plutei obtained by inseminating and rais-

ing the eggs in sea water to which various substances had been added. She found that HCl, CO_2 and acetic acid markedly retarded development. In view of Loeb's statement that alkali does not have any effect on the early development of *Arbacia,* she re-examined this point by counting the number of divided eggs one hour after insemination, and by comparing the skeletal development of the larvæ 18 hours after insemination. She found a definite acceleration from one to 18 hours with NaOH (greatest in 1.33 cc. *N*/10 NaOH + 98.66 cc. sea water) and with Na_2CO_3 (greatest in 0.4 cc. 0.45 *M.* Na_2CO_3 + 99.6 cc. sea water), though in later stages the alkali cultures showed a retardation so that ultimately they lagged behind the controls. Larger quantities of NaOH and Na_2CO_3 inhibited development from the beginning. NaCl produced slender, perforated skeletons with conspicuous processes; there was inhibition during early development and excessive growth during later periods. NaOH led to irregularity and asymmetry, while $NaHCO_3$ increased the bulk of the skeleton with a strong tendency for regularity and symmetry.

Richards (9) has recently observed acceleration of the early cleavage rate of the eggs of the opisthobranch, *Haminea virescens.* in sea water to which NaOH and KOH had been added. No acceleration was observed after the addition of $Ba(OH)_2$ and $Cr(OH)_3$.

In none of the investigations cited were the H-ion concentrations determined or controlled, nor was allowance made for any specific influence which the carbonic acid present in acidified sea water might have. Knowing that carbonic acid inhibits cell division at H-ion concentrations which otherwise are innocuous (10), it is important to determine the limits of reaction of CO_2-free sea water within which normal development is possible.

In performing the experiments reported in this paper, *Arbacia* and *Asterias* eggs were inseminated in sea water and subsequently transferred to 100 cc. of the pH solutions prepared as described in a previous paper (11). At appropriate intervals samples of 3 to 5 cc. were removed from each lot and fixed by the addition, in the case of Arbacia, of 2 or 3 cc. of a 1–1000 solution of formalin in sea water; the *Asterias* eggs were fixed by adding 2 or 3 cc. of a 1–1000 mercury bichloride solution in sea water. These meth-

ods of fixation stop all developmental processes at once and the cleavage planes remain clearly visible for many hours. Careful counts were then made on each sample, noting the number of eggs which were undivided and the number which were in each stage of division. By multiplying the number of two-cell eggs by one, the 4-cell eggs by two, the 8-cell eggs by 3, etc., and dividing the total number of divisions by the total number of eggs, the number of divisions per egg in each sample was determined. This figure is an arithmetic index of the degree of development, or if expressed in terms of time, of the velocity of development. By counting two to 3 hundred eggs in each sample, considerable accuracy can be obtained.

A particular culture of eggs will develop under constant conditions in sea water with a mean velocity that remains practically constant so long as the number of cleavages can be accurately counted. Certain individuals will be slower than the mean and others will be faster than the mean, expressing differences in viability or developmental capacity. Such differences may be interpreted from a statistical point of view to indicate the fluctuations which any individual may undergo, and the mean to represent the behavior of the average individual. The variations observed in the development of different cultures present many interesting features which we cannot discuss at this time. It should be pointed out, however, that for studies of developmental velocity under normal and abnormal conditions, the ideal condition is to have a maximum distribution of variants ("slow" eggs and "fast" eggs) so that development will progress over short intervals of time (*i.e.*, 15 to 20 minutes) in a uniform manner. Though this condition usually obtains, there are times in the season when the eggs are in such uniform physiological condition that they divide almost simultaneously. At such times the number of divisions per egg increases by abrupt steps. This circumstance can be alleviated by averaging two successive observations on each culture. For the present purposes it will suffice to consider the mean development during the entire period of observation.

The influence of reaction on the early developmental rate of normally fertilized *Asterias* and *Arbacia* eggs is shown in Fig. 1. The data summarized in this figure are taken from several experi-

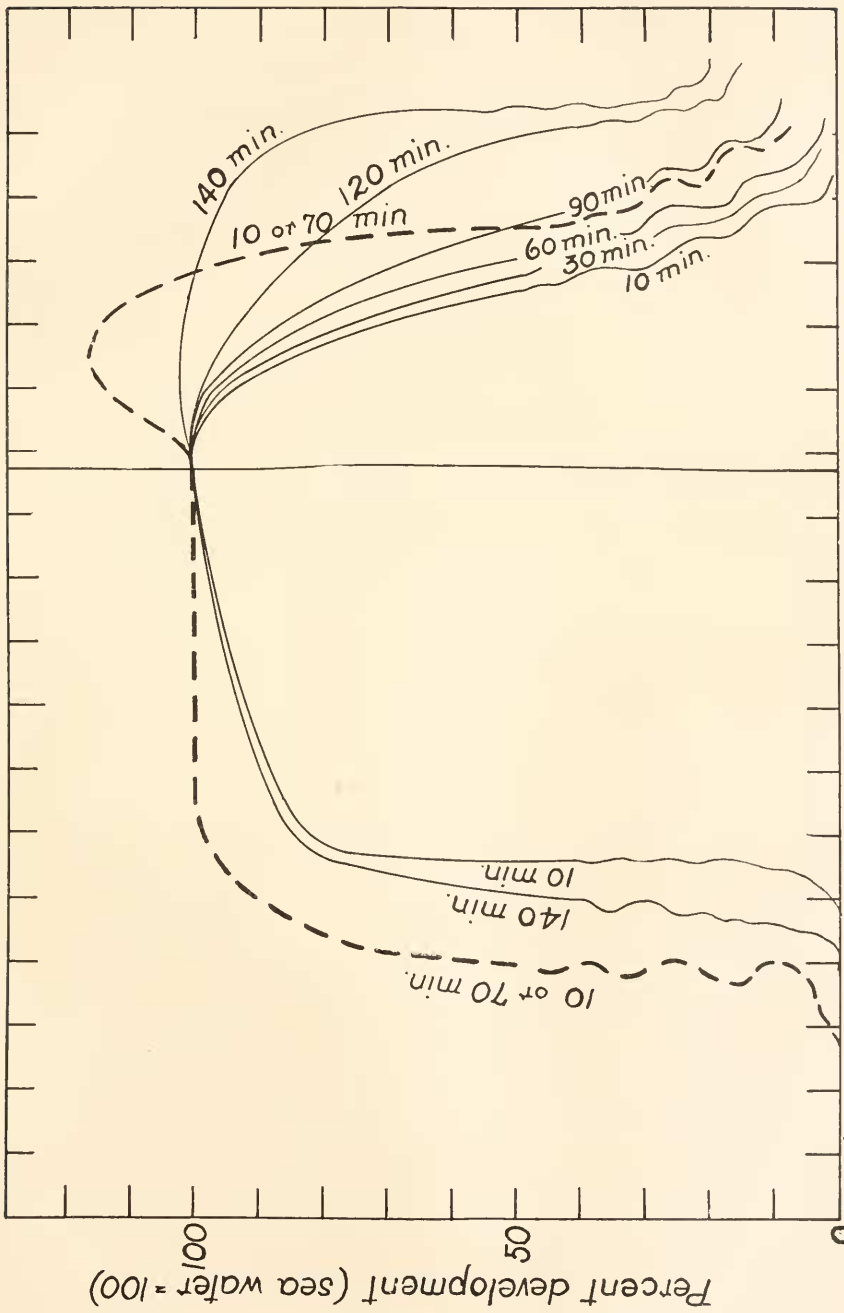

FIG. 1. The influence of H-ion concentration on the early development of normally fertilized *Arbacia* (dotted) and *Asterias* (solid line) eggs. The curves are based on the average results obtained from several experiments with each species and are expressed on the basis of the development in sea water (pH 8.15) as 100 per cent. The figures by each curve show the time after insemination at which the eggs were transferred from sea water to the acid and alkaline solutions.

ments with each species performed during the summers of 1922 and 1923. In these experiments the eggs were inseminated in sea water, centrifuged at various intervals after insemination and transferred to the pH solutions. The data given beside the curves show the time after insemination at which the eggs were transferred from sea water to the pH solutions. The development was followed quantitatively on samples taken every 20 minutes in which the number of divisions per egg was determined by careful counts. The mean development was then obtained by averaging all the observations for each solution, and the results expressed in terms of the corresponding figure for sea water as 100 per cent. The full ordinate indicates the H-ion concentration of sea water.

The dotted line in Fig. 1 refers to *Arbacia* and the solid lines to *Asterias*. The significance of the wavy portions of these lines will be discussed later. In *Arbacia* the velocity of division remains practically constant from pH 8.15 (the H-ion concentration of sea water) to pH 6.0; at 5.0 velocity of division is reduced by one half and at 4.6 division is completely suppressed. A slight increase in the alkalinity of sea water increases the velocity of division; this stimulation reaches its maximum about pH 8.8, and amounts to a 15 to 25 per cent. increase over the velocity in sea water. Above pH 8.8 there is an abrupt retardation so that the developmental velocity is reduced by one half at 9.6, and at 10.12 only a small fraction of the eggs divide even once.

Attention is called to the fact that the limiting reactions are characterized, not by a gradual, but by an abrupt inhibition of cell division within a comparatively narrow range. Between these limiting reactions cell division is essentially unimpaired.

It will be convenient for purposes of reference to define the critical limit as the pH at which the curve under consideration is reduced to its midpoint, *i.e.*, to 50 per cent. Accordingly the limits for *Arbacia* may be said to be pH 5.0 and 9.6. These limits are the same whether the eggs are placed in the pH solutions 10 minutes or 60 minutes after insemination. In the latter case, however, the degree of stimulation by alkali is slightly less.

The *Asterias* egg differs from the *Arbacia* egg notably in this—that while the resistance of the latter to both acid and alkali appears to be the same 10 minutes and 60 minutes after fertilization,

the resistance of the *Asterias* egg is much lower 10 minutes after fertilization than it is later on. There is a gradual increase in resistance, particularly in alkali, from 10 minutes after fertilization until the first cleavage, at which time the maximum resistance appears to be reached. Reference to Figure 1 will show that development is inhibited at pH 9.2 if the eggs are transferred to this solution 10 minutes after fertilization; but if the eggs are not transferred until the majority have reached the two cell stage (*i.e.*, about 140 minutes) they not only tolerate pH 9.2 but they tolerate equally well pH 10.0. This difference in resistance is strikingly shown by the cultures 24 hours later. When placed, for example, in pH 9.2 10 minutes after fertilization the velocity of development is greatly reduced; in many eggs the nucleus divides without cytoplasmic division; fragmentation and abortive division are predominant and no egg progresses beyond the 32-cell stage. When placed in this same solution after the first cleavage has occurred 90 per cent. of the eggs will develop to practically normal swimmers. The increase in resistance to acid is considerably less, the limiting acidity shortly after fertilization being pH 5.6, and 140 minutes after fertilization pH 5.4. The increase in resistance to alkali which gradually appears as the egg approaches the first cleavage plane is not to be confused with the period of great susceptibility which follows the event of fertilization, or with the periodic changes in resistance to various destructive agents which a number of observers have shown to be associated with the process of cleavage. The shortest interval after fertilization at which we transferred the eggs to the pH solutions was 10 minutes, and therefore the period of great susceptibility to destructive agents immediately following fertilization was avoided. And since in our experiments the eggs are left in the pH solutions until the conclusion of the experiments, which cover in the case of *Asterias* 5 cleavages, any periodic fluctuations in resistance, if they occur, are translated into a mean.

There is little increase in the velocity of division in alkaline solution, 2 or 3 per cent. being the maximum observed in any of our experiments. There is frequently a marked stimulation shortly after transferring to the alkaline solution, but this is transient and is followed by a decrease in the velocity of division, so

that after 4 or 5 cleavages the alkali cultures are about even with the controls. There is a slight but perceptible decrease of the velocity of division in acid solutions between pH 5.8 and 7.8, in contrast to the *Arbacia* egg where the velocity remains practically constant.

If we consider the limits for eggs transferred to the pH solutions at the time of the first cleavage, these limits are pH 5.4 and 10.1. Thus the limits within which the development of *Asterias* eggs is possible are distinctly on the alkaline side of those for *Arbacia* eggs.

In those solutions in which the velocity of development is reduced below 50 per cent., the quality of cell division in both *Arbacia* and *Asterias* eggs is greatly altered. The division of the cytoplasm is apparently restrained before the division of the nucleus, and in consequence the majority of eggs become multinuclear. This condition of abnormal division is indicated in Fig. 1 by the wavy portions of the curves. After two or 3 cleavages of the nucleus without cytoplasmic division the egg usually divides abruptly into more than two blastomeres, but the division is invariably abnormal and either soon ceases entirely or leads to cytolysis. In some cases it can be observed that the cytoplasm begins to divide but the furrow melts and the blastomeres fuse. The tendency for nuclear division without cytoplasmic division is much more marked in alkaline than in acid solutions. A point is reached on the alkaline side, however, where nuclear as well as cytoplasmic division is completely inhibited. A similar repression of cytoplasmic division without complete repression of nuclear division has been observed with lack of oxygen, the action of chloroform and ether, the action of hypertonic and hypotonic sea water, cold and other agents (12).

We are concerned here principally with variations in developmental velocity which are made manifest in the early history of the dividing egg, during that period of time in which accurate quantitative information can be obtained. It is of interest to consider, however, the effects of longer exposures. A method is not available for expressing these effects quantitatively but a fair idea of the degree of retardation during a 24 hour exposure can be obtained by comparing the general development of the larvæ.

Such comparisons have shown that some retardation of development occurs even at pH 7.6 and 8.5, and that the effect of increasing acidity or alkalinity does not take the form of an abrupt inhibition at any point, but manifests itself in almost imperceptible gradations from normal development to no development at all. In the acid solutions the inhibition culminates in coagulation with little division; and in alkaline solutions in either complete cytolysis or in the formation of formless, ciliated masses of protoplasm swimming within the fertilization membrane. It is doubtful if normal development can be obtained throughout a period of 24 hours in solutions more acid than pH 7.8, or more alkaline than 8.4.

Summary.

The effect of acid and alkaline sea water on the rate of cell division in normally fertilized *Arbacia* and *Asterias* eggs was observed as far as the 128-cell stage.

In *Arbacia,* the velocity of division is reduced to 50 per cent. of the velocity in sea water (pH 8.15) at pH 5.2 and 9.4. Between pH 5.8 and 8.2 these eggs divide normally both in respect to velocity and quality of cell division. Between pH 8.2 and 9.2 the velocity of division is increased from 15 to 25 per cent.

Asterias eggs are more sensitive to both acid and alkaline sea water during the precleavage period than at any subsequent time. When these eggs are transferred to the acid and alkaline sea water immediately after fertilization, the velocity of division is reduced to 50 per cent. at pH 5.6 and 9.2; when transferred in the two cell stage the corresponding limits are pH 5.4 and 10.2. There is a slight decrease in the mean velocity of division between pH 8.2 and 5.8, but no significant increase in solutions more alkaline than sea water.

In both species, when the developmental velocity is reduced below 50 per cent. by either acid or alkali, the nucleus tends to divide without division of the cytoplasm, and abnormal multinuclear cells are formed.

We are indebted to Mabel T. Studebaker for the statistical work in the experiments recorded in this paper.

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THE INFLUENCE OF HYDROGEN ION CONCENTRATION ON THE FERTILIZATION PROCESS IN *ARBACIA, ASTERIAS* AND *CHÆTOPTERUS* EGGS.

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In a previous communication we pointed out that when *Asterias* and *Arbacia* eggs were inseminated in CO_2-free sea water of varying H-ion concentration, fertilization failed to occur in solutions more acid than pH 6.6 to 7.0. This block to fertilization appeared to be perfectly reversible, since eggs which did not fertilize in solutions on the acid side of the block could be fertilized when returned to solutions of greater alkalinity (1). Loeb (2) has observed a similar block to fertilization in artificial salt solutions. He found that *Arbacia* and *S. purpuratus* eggs were not fertilized in a neutral mixture of $NaCl + MgCl_2$ in the proportion in which these salts exist in sea water. These eggs were fertilized, however, if NaOH, NH_4OH, benzylamine, butylamine or $NaHCO_3$ were added to the $NaCl + MgCl_2$ mixture. The addition of $CaCl_2$ to the $NaCl + MgCl_2$ mixture similarly made fertilization possible. The addition of NaOH or $CaCl_2$ to a $NaCl + KCl$ mixture did not permit fertilization of all eggs, but when both NaOH and $CaCl_2$ were added to a $NaCl + KCl$ mixture as a rule all the eggs fertilized and began to divide. Since cross fertilization can be effected between *Asterias* sperm and *S. purpuratus* eggs by the addition of NaOH or $CaCl_2$ to normal sea water, Loeb concluded that the act of diminishing the alkalinity of the solution or of depriving it of $CaCl_2$ established the same reversible block to the entrance of the homologous sperm as exists for the entrance of the sperm of *Asterias* into *S. purpuratus* eggs in normal sea water. Leob's experiments involve the change of several variables at once, however, and it cannot be determined from them to what extent the reaction of the external medium

per se influences the fertilization of eggs by sperm of the same species.

Further examination of the block to fertilization which is created when the H-ion concentration of sea water is increased to a critical point has convinced us of its physiological significance, and we have extended our observations to include the effects of increasing alkalinity on the fertilization of *Arbacia* and *Asterias* eggs, and the effects of acid and alkaline sea water on the fertilization of the eggs of *Chætopterus pergamentaceous*.

The Acid Block to Fertilization.

CO_2-free sea water solutions were prepared as described in a previous paper (3). Our experiments on fertilization were performed as follows: A drop of concentrated egg suspension was added to 50 or 100 cc. of each of the pH solutions, and a drop of sperm suspension was added to about 5 cc. of the pH solutions. After an interval of 3 to 5 minutes the sperm and eggs were mixed and thoroughly agitated. (No precautions were taken to remove body fluids which might be present around the eggs, other than the routine washing which they were always given in preparing them for any experiment.) Subsequently the proportion of fertilized eggs in each dish was carefully determined. It makes no difference whether the counts are made 10 minutes or several hours after insemination because every egg that is going to fertilize will lift a membrane within the normal time of 3 to 5 minutes. It has been our custom in performing experiments of this kind to remove samples from the pH solutions 10 or 15 minutes after insemination and return them to sea water with fresh sperm to make sure that the eggs had not been irreversibly modified by the action of the pH solutions or by contact with sperm in these solutions. It may be said that this procedure has one invariable result; if the exposure is below that required for the acid to injure the egg, then every egg which is not fertilized on the acid side of the block will fertilize when returned to sea water with fresh sperm.

The influence of H-ion concentration on the fertilization of *Arbacia* and *Asterias* is illustrated in Figs. 1 and 2. The solid line in each figure indicates the range within which fertilization

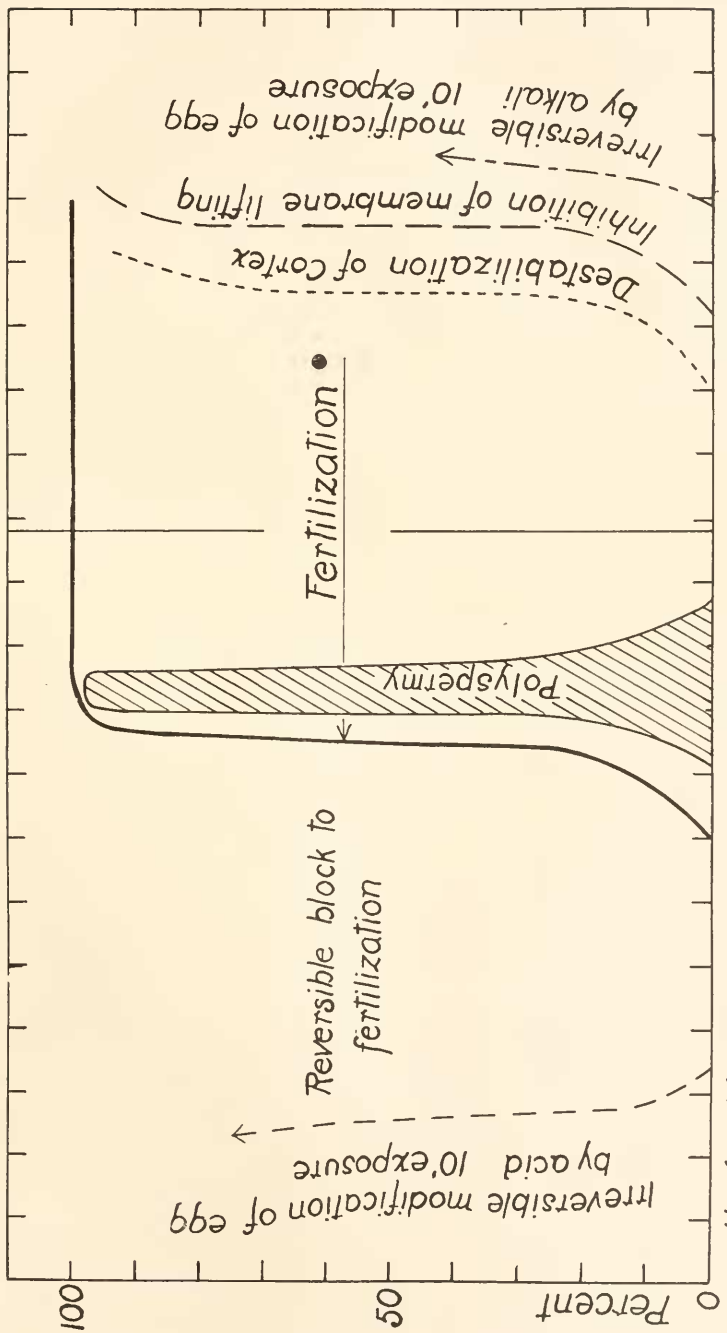

FIG. 1. The influence of H-ion concentration on the fertilization of *Arbacia* eggs.

occurs. The cessation of fertilization with increasing acidity is very abrupt, but the critical H-ion concentration may be most accurately indicated by the pH at which only 50 per cent. of the eggs fertilize. This critical H-ion concentration is pH 6.8 for *Arbacia* eggs, and 7.0 for *Asterias* eggs. In solutions slightly more alkaline than these, all the eggs fertilize; and in solutions slightly more

TABLE I.

A. ACID BLOCK TO FERTILIZATION IN *Asterias*.

Per Cent. of Eggs Fertilized:

pH									Date.
6.6	6.7	6.8	6.9	7.0	7.1	7.2	7.3	7.4	
0		0		50		100		100	Conclusions 1921.
0		1		42		100			6–30–22
0		0		21		85			7– 6–22
0		0		0		4		76	5–29–23
0		0	1	5	5	40		72	5–30–23
0	0	0	0	47	60	85		93	8–31–23
0	0	0	0	0		82		100	8–31–23
0	0	0	0	60		100		100	8–31–23
0	0	0	10	6		21		72	9– 2–23
0	0	0	14	11		22		90	9– 3–23
0	0	0	40	40		100		100	9– 3–23

B. ACID BLOCK TO FERTILIZATION IN *Arbacia*.

Per Cent. of Eggs Fertilized.

pH									Date.
6.6	6.7	6.8	6.9	7.0	7.1	7.2	7.3	7.4	
0		50		100		100		100	Results of 1921.
2		47		98		98		97	6–30–22
0	0	100	100	100	100	100		100	7–20–23
0	0	30	20	75	100				9–21–23
2	7	28	24	95	100	100	100		9–22–23
7	29	100	100	100	100	100	100	100	9–22–23
0	0	30	20	75	100	100	100	100	9–22–23

C. COMPARISON OF ACID BLOCK IN *Asterias* AND *Arbacia* IN SAME pH SOLUTIONS. 9–21–23.

Per Cent. of Eggs Fertilized.

	pH								
	6.6	6.7	6.8	6.9	7.0	7.1	7.2	7.3	7.4
Asterias	0	0	0	1	30	100	100	100	100
Arbacia	5	0	30	70	100	100	100	100	100

acid, none of the eggs fertilize. We have previously shown that the unfertilized eggs of these species are uninjured by short exposures to the solutions in which fertilization does not occur (3); and that normally fertilized eggs will develop with normal velocity at H-ion concentrations much greater than those at which the block to fertilization occurs (4).

The constancy of this acid block to fertilization is very marked. To illustrate this a few experiments have been given below. Table I. contains a résumé of experiments performed in 1921, 1922 and 1923 on the fertilization of *Asterias* and *Arbacia* eggs. Despite the probability of variable conditions in these experiments, the point at which 50 per cent. of the eggs fertilized remained constant to $\pm$ 0.2 pH.

There are at least two factors which might be expected to shift the block one direction or another; first, the length of time which the eggs or sperm have remained in the acid solution, and second, the relative quantity of sperm used for insemination. Examination of the first factor has shown that the equilibrium between the pH solution and the egg (or sperm) is reached with astonishing rapidity. This can be illustrated by first adding the sperm to the pH solution and then adding to the resulting sperm suspension a drop of eggs suspended in sea water. Under these conditions one would expect that the time required for the egg cortex to come to chemical equilibrium with the pH solution would be long enough to permit many more eggs to be reached by sperm and fertilized than would be the case if the eggs were allowed to come to equilibrium with the solution before adding the sperm. The results of experiments of this kind with *Asterias* eggs are given in Table II. Converse experiments were simultaneously performed; the eggs were added to the pH solution first and after 5 minutes a drop of comparatively concentrated sperm suspended in sea water was added to these eggs. When the experiment is performed as first described, the block appears at the same pH as when both eggs and sperm are at equilibrium with the pH solutions before insemination. When the experiment is reversed, the block is shifted slightly towards the alkaline side. This indicates that the essential equilibrium underlying the block involves the egg cortex rather than the sperm. The difference is hardly great

enough to be significant, though the results do show very definitely that chemical equilibrium between either eggs or sperm and the pH solutions is reached in less time than is required for sperm to reach the eggs and fertilize them.

TABLE II.

A. EFFECT OF EXPOSING *Asterias* SPERM TO pH SOLUTIONS FOR 5 MINUTES BEFORE ADDING *Asterias* EGGS IN SEA WATER. 8–31–23.

Per Cent. Eggs Fertilized.

pH									
6.2	6.6	6.8	6.9	7.0	7.1	7.2	7.6	8.15	
0	0	0	6	8	81	85	90	83	(Exp. 1)
0	0	0	0	47	85	90	89	97	(Exp. 2)

B. EFFECT OF EXPOSING *Asterias* EGGS TO pH SOLUTIONS FOR 5 MINUTES BEFORE ADDING *Asterias* SPERM IN SEA WATER. 8–31–23.

Per Cent. Eggs Fertilized.

pH								
6.2	6.6	6.8	6.9	7.0	7.1	7.2	7.6	
0	0	0	0	0	49	82	100	(Exp. 1)
0	0	0	0	2	63	95	100	(Exp. 2)

One of several experiments testing the influence of varying quantities of sperm in shifting the limits of fertilization is given in Table III. The eggs were placed in the pH solutions and 5 minutes later the sperm, diluted with the pH solutions, were added.

TABLE III.

INFLUENCE OF QUANTITY OF SPERM ON ACID BLOCK IN *Asterias*.

Per Cent. Eggs Fertilized.

pH										Quantity of sperm added to 25 cc. pH sol.
6.6	6.7	6.8	6.9	7.0	7.1	7.2	7.3	7.4	8.15	
0	0	0	50	100	95	95	100	100	100	1 cc. 1–20
0	0	0	0	35	80	95	100	100	100	1 cc. 1–200
0	0	0	0	0	20	85	87	95	90	1 cc. 1–2,000
0	0	0	0	1	40	45	25	35	30	1 cc. 1–20,000

The smallest quantity of sperm was insufficient to fertilize all the eggs even in sea water, and the largest quantity gave a dis-

tinctly opalescent suspension; yet the block did not shift beyond the limits pH 6.9 to 7.1. In general, increasing the quantity of sperm used in insemination increases the proportion of eggs fertilized in the acid solutions, but the shift to the acid side is not so great as would be expected if the failure to fertilize in the acid solutions were attributable to an impairment of the sperm. Rather the slight magnitude of this shift favors the belief that the block is due to an alteration of the properties of the egg.

It may be stated here that unless the sperm are injured or attenuated by the toxic action of egg secretions, all eggs which fertilize in the pH solutions develop normally, indicating that the fertilization reaction when once initiated in the neighborhood of the block, is completed without impairment.

The Acid Block in *Chætopterus*.

The determination of the acid block to fertilization in *Chætopterus* was made in the same manner as in *Asterias* and *Arbacia*. The egg sacks were cut in sea water and the eggs liberated from the ovaries by teasing these to pieces. The ovary fragments were removed by straining through cheese cloth, and when the eggs had maturated they were concentrated by centrifuging. A drop of the concentrated egg suspension was added to 50 cc. of the pH solution; after 5 minutes the sperm, previously diluted with the pH solutions, were added and the mixture agitated. The per cent. of fertilized eggs was determined by counting the dividing eggs one and a half to four hours after insemination.

The scarcity of material made it impossible to get more than a half dozen determinations; of these, two were discarded since only a small proportion of the eggs were fertilized in sea water. The remaining four indicated that the block appeared between pH 7.0 and 7.3, and from the two most satisfactory experiments the block was tentatively set at pH 7.1.

The acid (pH 5.8) activation of the *Chætopterus* egg, with the consequent temporary block to fertilization, has been discussed in a previous paper (3). This block, which is most effectually established by short exposures to pH 5.2 to 6.4, was tentatively ascribed to cortical changes which tend to persist after the eggs have been removed from the acid solutions, and returned to sea water. It is in no sense comparable to the physiological block occurring at

pH 7.1; the latter is perfectly and instantly reversible, as in *Asterias* and *Arbacia*, disappearing as soon as the eggs are returned to a more alkaline solution.

THE INFLUENCE OF ALKALI ON THE FERTILIZATION REACTION.

Frank Lillie (5) has observed that the addition of alkali to the sea water in which insemination occurs increases the incidence of fertilization in *Asterias* and *Arbacia* eggs of poor quality. We have confirmed this in *Asterias* and *Arbacia* and found that it is equally true for *Chætopterus*. This effect of a slight increase in alkalinity in aiding fertilization may be due to action on the sperm but it seems more probable that both the eggs and the sperm are affected. The changes which culminate in increased fluidity of the egg cortex in alkaline sea water (3) are no doubt preceded by enhanced physiological reactivity.

Apart from this stimulating action of alkali, which is not apparent in eggs of the best quality, fertilization in *Asterias* and *Arbacia* proceeds unimpaired from pH 8.15 to 9.6. With further increases in alkalinity, eggs appear in increasing numbers which have either tight or incompletely formed fertilization membranes; and at pH 10.2 the eggs have no demonstrable membranes at all. When returned to sea water after a 3 to 5 minute exposure to pH 10.0, fertilization membranes will form on most of the previously unmembraned eggs. It was concluded that these eggs were fertilized while in the alkaline solution, since the supernatant sperm carried over from the alkaline solution are incapable of fertilizing fresh eggs. Longer exposures injure the eggs to such an extent that membranes do not form on them when they are returned to sea water. The H-ion concentrations which prevent membrane elevation (and which destabilize the cortex of the unfertilized egg (4)) are shown in Figs. 1 and 2 by the dotted lines at the extreme right.

This alkaline injury is more rapid in *Asterias* than in *Arbacia*. In the latter case the eggs will divide imperfectly if returned to sea water after a 5 to 10 minute exposure to the alkaline solution. In *Asterias* the inhibition of membrane elevation is rapidly followed by a more profound injury which completely stops development. Such eggs can not be fertilized by fresh sperm in sea water.

These facts all indicate that under increased alkalinity union of the egg and sperm still occurs; but if the increased alkalinity

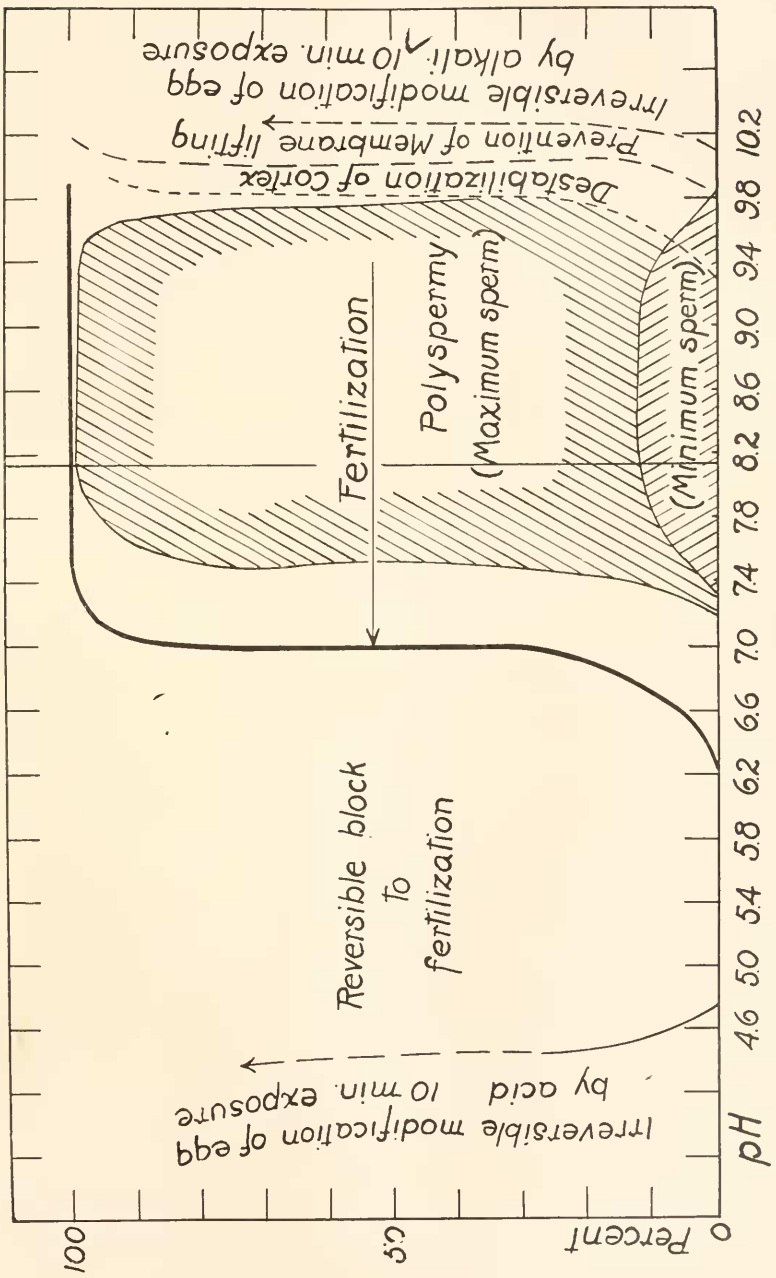

FIG. 2. The influence of H-ion concentration on the fertilization of *Asterias* eggs.

does not actually introduce some abnormality into this initial event, it impairs subsequent events of the fertilization process to such a degree as to prevent normal development. There is apparently no reversible block created by alkali corresponding to that created by acid, where the fertilization reaction proceeds in an all-or-none fashion. This conclusion is supported by the extremely rapid injury of the eggs and sperm if separately exposed to the alkaline solutions (pH 10.0), which prevent normal fertilization, and the complete absence of such injury in acid solutions (pH 6.8 to 7.0) which have a similar effect.

Polyspermy.

In the case of *Arbacia* eggs there is a very narrow range in H-ion concentration in which the incidence of polyspermy is unusually high. This range is approximately defined in Figure 1 by the heavily shaded portion; the maximum of polyspermy is close to pH 7.2. Though the incidence of polyspermy at all H-ion concentrations increases with increasing age or staleing of the eggs, yet within this narrow range, centering at pH 7.2, practically all the eggs will be polyspermic even when they are fresh and when the incidence of polyspermy is nearly zero from pH 7.4 to 9.8.

In *Asterias,* polyspermy shows no marked maximum at any H-ion concentration but occurs more or less uniformly from pH 8.5 to 9.5 (Fig. 2). When excessive quantities of sperm are used in insemination, nearly all the eggs may be polyspermic from 8.15 to 9.6. It is perhaps significant that the polyspermy curve, even though extremely broad, is limited on the alkaline side; for the incidence of polyspermy decreases appreciably before the alkalinity is sufficient to inhibit fertilization, indicating that in its general nature the underlying mechanism in *Asterias* is similar to that in *Arbacia.*

We did not have the opportunity to make similar observations on polyspermy in *Chætopterus.* Such data as we have indicate that there is, as in *Arbacia,* a comparatively narrow region in which polyspermy predominates (about pH 9.5).

Reaction of Sperm with Immature *Asterias* Eggs.

When immature *Asterias* eggs are inseminated in sea water, several sperm usually enter each egg before the fertilization membrane is formed. Subsequently the germinal vesicle breaks down and the cytoplasm acquires a mottled appearance, each sperm being the focus of a localized cytolytic process. Such prematurely fertilized eggs never attempt to divide. If sperm are added to immature eggs at various H-ion concentrations, a block appears at the same point as in the fertilization of the mature egg, viz., pH 7.0. On the alkaline side of this point the sperm enter the eggs, causing membrane elevation and the changes described above. On the acid side the sperm do not react with the eggs in any way; in the course of time, a varying proportion of these unfertilized eggs will maturate, depending on the H-ion concentration, and these, if they are returned to sea water and inseminated, will fertilize and develop normally.

Summary.

When *Arbacia, Asterias* and *Chætopterus* eggs are inseminated in CO_2-free sea water of varying H-ion concentration, a block to fertilization appears at a H-ion concentration which is constant, and apparently characteristic for each species. If the block is defined by the H-ion concentration at which 50 per cent. of the eggs fertilize, these H-ion concentrations are: *Arbacia,* pH 6.8; *Asterias,* pH 7.0; and *Chætopterus,* pH 7.1.

This block to fertilization is complete, in that eggs either fertilize and develop normally, or do not fertilize at all; and it is perfectly reversible, in that eggs which do not fertilize on the acid side of the block will fertilize immediately if they are returned to solutions on the alkaline side of the block and inseminated with fresh sperm.

In sea water more alkaline than pH 9.8 to 10.0 the fertilization process in both *Arbacia* and *Asterias* eggs is either incomplete or impaired. Apparently there is no alkaline block to fertilization corresponding in its complete reversibility to the block which appears around neutrality.

In *Arbacia* there is an increased incidence of polyspermy within a very narrow range centering at pH 7.2, indicating some critical

condition in the mechanism of fertilization at this H-ion concentration. In *Asterias* polyspermy occurs more or less uniformly over a wide range extending from pH 7.2 to 9.8.

We are indebted to Mabel T. Studebaker for the statistical work in the experiments recorded in this paper.

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HERMAPHRODITISM IN *EURYCEA BISLINEATA*.[1]

INEZ WHIPPLE WILDER AND ELIZABETH BARRETT PEABODY.

The occurrence of hermaphroditism among anurans seems to be an accepted fact. Crew ('21) summarized all the recorded cases of abnormal sexual organs in frogs and states that there are forty such cases. To this Swingle ('22) has recently added one more, but finds that of his list of forty-one abnormalties only twenty-seven can be considered hermaphrodites, a sufficient number, however, coming from the hands of so severe a critic, to warrant the statement that hermaphroditism in anurans does occur. Cerruti ('07) and King ('10) following numerous earlier writers, have investigated the occurrence of the anomaly in toads with results which, though possibly subject to differences in interpretation, tend nevertheless to substantiate the existence of hermaphroditism in these forms.

No one has done for the urodeles the service which Crew has performed for the frogs, but from the paucity of published reports upon anomalies in urodeles this would not seem to be an arduous task. Thus Chapin ('15) in reporting a case of hermaphroditism found by her in *Spelerpes bislineatus* (*Eurycea bislineata*) cited reports of only two other cases of this anomaly in urodeles which had come to her attention, one that of La Valette St. George ('95) in *Triton tæniatus*, the other that of Knappe ('86) in a young *Salamandra maculata*. Since the publication of Chapin's paper a third case has been reported by Krizenecky ('17) in *Triton cristatus*. Although the cases of La Valette St. George and of Křizenecky in *Triton* are unquestionably to be accepted as genuine, there is doubt concerning the nature of the anomaly reported by Knappe in *Salamandra*. Its interpretation as an hermaphrodite is apparently that of King ('10) who in summing up the reported cases of the occurrence of hermaphroditism in urodeles says that "Knappe ('86) noted the presence of a Bidder's organ in a young salamander." In the paper in question, however, following the enumeration of the species of Amphibia which he

[1] Contribution from the Department of Zoölogy of Smith College, No. 116.

had examined for the possible occurrence of a Bidder's organ, an enumeration which included seven species of *Anura* and two of *Urodela* (*Triton tæniatus* and *Salamandra maculata*) Knappe states definitely "Bald stellte sich heraus, dass es zur Bildung eines Bidder'schen Organs nur bei den echten Krötenarten kommt." And farther on after mentioning the unique appearance of one Bidder's organ, he says: "Eine solche Samenkörperbildung in Eikapseln des Bidder'schen Organs mit gleiches Bestimmtheit nachzuweisen, wie in dem eben beschriebenen Falle, ist mir bis jetzt nicht wieder gelungen, doch kann ich ähnliches für eine andere verwandte Thiergruppe, die Salamander, konstatiren. So liess eine in Schnittserien zerlegte Hodenabtheilung eines jungen, vielliclit zweijährigen Salamandra maculata nicht den geringsten Zweifel, dass dieselbe aus Eikapseln, ähnlich denen im Bidder'schen Organ der Kröten, bestäht." It was thus obviously not Knappe's intention to state that he found a Bidder's organ in a salamander, but rather an appearance in the testis of a salamander like that of the unique Bidder's organ in a toad. In any case the interpretation of the condition described in the salamander as hermaphroditic, will depend upon the interpretation of the sexual nature of Bidder's organ itself. This is a matter which has been a bone of contention ever since the discovery of the organ in 1758 by Rösel von Rosenhof, and a number of theories have been advanced regarding its nature and significance.

In view of the almost universal agreement of modern writers as to the femaleness of Bidder's organ, Swingle's recent discussion ('21 and '22) of its nature is of great importance. In a discussion of the so-called transformation of sex in frogs, he claims that the theory is really based on a misinterpretation of the appearance of the cells in the Bidder's organ of toads. According to Swingle, the oviform-like cells of this organ do not represent the cells of an ovary, thus making the animal an hermaphrodite at this stage, but are, like the cells of similar appearance which occur in the protestis of the frogs, merely senescent male cells which are undergoing oviform degeneration. He adds further: "True hermaphroditism in frogs is a permanent and pathological condition, probably due to a mix-up in the genetic constitution of the individual,

and is not to be confused with the present problem which has to do with a normal but transitory embryological process."

The general opinion thus set forth by Swingle finds support also in a statement made by Crew ('21) who said: "Cytologically it has not been proved that the cells which constitute Bidder's organ are ovarian and there undoubtedly are reasons for questioning the generally accepted opinion that this organ is a rudimentary ovary."

The three cases of hermaphroditism already reported in urodeles, disregarding now Knappe's inconclusive report, differ from each other quite markedly. La Valette St. George's case in *Triton tæniatus* was referred to by Cole ('96) as "the most complete case of hermaphroditism yet recorded among the Amphibia." The specimen was a male with perfectly distinct and independent paired ovaries, in addition to a pair of normal testes, but, however, without any traces of oviducts. The testes contained developing and fully developed sperms; and the ovaries, eggs in various stages of maturity.

The case reported by Křizenecky in *Triton cristatus* showed the presence of ova within both peripheral and internal lobules of otherwise normal testes.

The case reported by Chapin in *Eurycea bislineata* was that of an advanced larva in which the gonad was essentially male with female elements. Macroscopically, the anterior part of the left gonad, which was much reduced in size, resembled the normal testis in texture, though not in shape, while the posterior region was distinctly like an ovary. The right gonad, which was somewhat smaller than the normal testis of an individual of the same size, showed another sort of hermaphroditism. Two ova were found in the otherwise apparently normal testis, each one completely filling one lobule, which would normally contain a large number of male cells. This case showed, therefore, two ways in which female elements may be disposed in otherwise distinctly male gonads; one in the form of growing ova among the cysts of spermatogonia, and the other by a modification of a part of the gonad into a region resembling an ovary. The numerous cases of hermaphroditism which we have found in this same species are all of the same general character as that described by Chapin.

In the light of a recent article by Jordan ('22), it may be well to define our use of the term, hermaphroditism. According to Jordan, true anatomic hermaphroditism occurs "where ovary and testis are present in the same individual." Jordan regards the presence of an ovo-testis as a modification of true hermaphroditism, a condition which he designates as a type of false hermaphro-

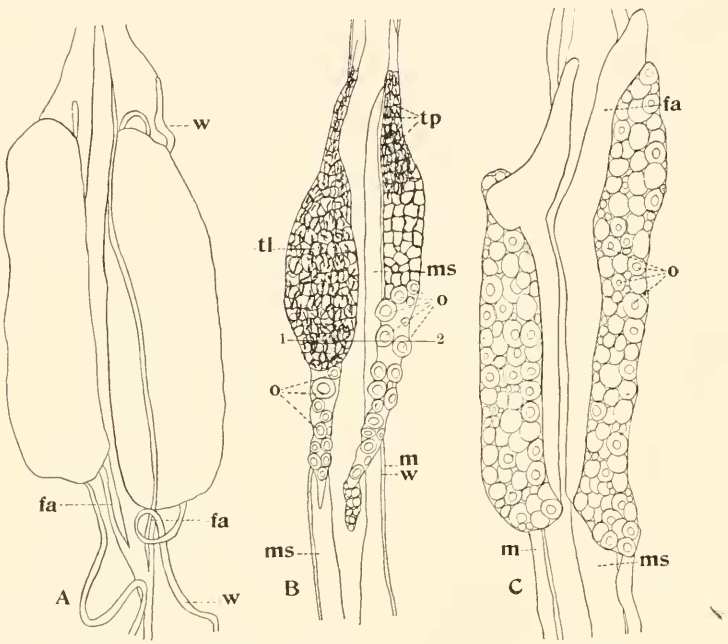

FIG. 1. Camera lucida drawings of the ventral view of the gonads of (*A*) an adult male; (*B*) an adult hermaphrodite; and (*C*) an adult female (× 9). *Fa*, fat bodies; *m*, Müllerian duct; *ms*, mesonephros; *o*, ova (primary oöcytes). *tl*, testicular lobules; *tp*, testicular pigmentation (the two latter present but not shown in (*A*)); *w*, Wolffian duct. The line 1–2 shows the level of the section drawn in Fig. 4.

ditism. Were this distinction to be accepted, the term, true hermaphroditism, could be used only when referring to such a case as that of La Valette St. George's in *Triton tæniatus*. There seems to be no justification, however, for this distinction of Jordan's, inasmuch as a distinct testis and ovary is but a further step in the separation of the male and female elements which, in some individuals, are still intermingled to a greater or less extent in the ovo-testis. An examination of the adult ovo-testis shown in

Fig. 1 *B* shows that a separation posterior to the testicular part on each side, such as, in fact, is slightly indicated in the right gonad, would transform each ovo-testis into a distinct testis and ovary. A female was found in which the ovary (Fig. 2) showed a number of separate parts or lobes, some connected with each other by

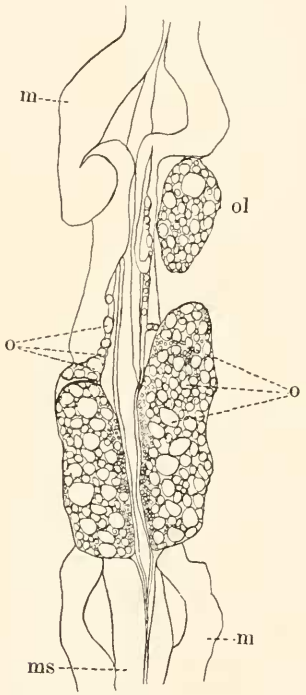

Fig. 2. Camera lucida drawing of the ovaries of an adult female showing an unusual lobed form (× 7). *M*, Müllerian duct; *ms*, mesonephros; *o*, ova; *ol*, detached lobe of ovary.

the mesovarium and others quite distinct, serving to illustrate the point that parts which are usually continuous may, through some unknown cause, become thus carried apart. Jordan's distinction in terminology seems, therefore, a somewhat arbitrary one, at least as applied to our species, and thus any individual which shows the presence of both male and female sex cells, even though these appear side by side in the same gonad, is regarded in this paper as a true hermaphrodite.

Naturally the ultimate criterion of hermaphroditism should be the production of functional germ cells of both sexes. Such a

criterion would obviate all possibility of the condition being given the interpretation which Swingle has given to the oviform cells which occur in Bidder's organ and in the larval testis (pro-testis) of frogs. In carrying out our investigation we had in mind as near an approach to this ideal as possible, and, having found examples of the condition which to us seemed unquestionably hermaphroditic in individuals of various stages up to transformation, we made a definite search for such cases among adult animals. As this search was rewarded by the discovery of one adult in which the hermaphroditic condition was beyond question, although the individual had not arrived at full sexual maturity, we feel confident that our interpretation of our cases as true hermaphrodites is correct and that the condition described cannot be considered as "a normal but transitory embryological process."

Percentage of Occurrence.

The determination of the percentage of occurrence of hermaphrodites with reference to that of males and females in *Eurycea bislineata* is based upon the examination of the gonads of 1113 individuals ranging from the typical larval to the adult stage. Wilder ('24) has shown that *Eurycea bislineata* is a form in which the period before transformation is considerably prolonged, covering from two to three years, although the structural changes leading toward metamorphosis are inaugurated many months previous to the actual transformation. The whole period from hatching to transformation is subdivided on the basis of structural changes into stages, the readily recognizable criteria of which, in living individuals, are as follows:

1. Postembryonic stage—Yolk still present, intestine not fully formed.
2. Typical larval stage—Intestine fully formed; no naso-lacrimal groove and no *os thyreoideum.*
3. Premetamorphic stage—Open naso-lacrimal groove (in incipient phase); *os thyreoideum* present; no vesicular glands in the skin.
4. Metamorphic stage—Glands of skin appearing as tiny acinous vesicles (in incipient phase), becoming rapidly larger and more conspicuous; absorption of larval structures and de-

velopment of eyelids and naso-labial groove (advanced phase).

The specimens used constituted representative collections made through a period of several years and had been preserved either in alcohol after fixation in Bouin's solution, or in formalin.

The method followed in sexing was first to examine the gonads *in situ* under a Bausch and Lomb binocular dissecting microscope with a strong artificial illumination. In many cases this was sufficient to diagnose the sex, but in those cases in which it was not, the gonad was removed and cleared *in toto* in glycerine for more careful study under the compound microscope. If in sexing one begins with adults and continues through the smaller and earlier stages, one comes finally to a point where it is practically impossible to be sure of the sex. Individuals of less than 27 mm., though frequently possessing readily sexed gonads, more often exhibit a developmental condition which might admit of various interpretations, since, at least without the use of cytological criteria, the small cells present might be either oögonia or spermatogonia. Bouin ('01) found that in *Rana temporaria* the first development of male and female germ-cells is identical as far as origin and general appearance are concerned. It may even be the case in *Eurycea,* as Okkelberg ('21) has shown for the brook lamprey, that the animal passes through a period of sex indifference before sex differentiation sets in. His observations "seem to warrant the conclusion that each larva of this species (*Entosphenus wilderi*) carries the potentiality of both sexes and that sex, therefore, is not irrevocably fixed at fertilization." He explains the development of sex in these gonads of "potentially either" sex by showing the presence in the gland of two kinds of germ cells, those manifesting a tendency towards rapid division (katabolic) and those showing a tendency to growth (anabolic). He says: "The former are regarded as having a male and the latter a female potentiality. The relative proportion of anabolic and katabolic cells determines whether the larva becomes a male or a female individual."

Since our problem, however, was not one dealing with a possible early transitory hermaphroditic condition, we have included in the calculation of the percentage of occurrence only those indi-

viduals which had definitely passed beyond the indeterminate sexual stage to a point where sex could be definitely diagnosed. The following summary is, therefore, based upon the study of gonads of animals of a length of 27 mm. and over, the arbitrary minimum of 27 mm. being taken as approximately representing the dividing line between those individuals in which the sex is still, if not indifferent, at least frequently difficult to determine, and those in which the sex is unquestionably established and recognizable.

Summary of Percentage of Occurrence.

Typical larval individuals (of 27 mm. and over in length)

Total number sexed	178	
Number of males	86,	or 48.3 %
Number of females	89,	or 50.0 %
Number of hermaphrodites	3,	or 1.68%

Individuals in incipient phase of premetamorphosis

Total number sexed	333	
Number of males	177,	or 53.2 %
Number of females	152,	or 45.7 %
Number of hermaphrodites	4,	or 1.2 %

Premetamorphic individuals (beyond incipient phase)

Total number sexed	256	
Number of males	119,	or 46.5 %
Number of females	134,	or 52.3 %
Number of hermaphrodites	3,	or 1.2 %

Metamorphic individuals

Total number sexed	226	
Number of males	115,	or 50.8 %
Number of females	107,	or 47.4 %
Number of hermaphrodites	4,	or 1.8 %

Adult individuals

Total number sexed	120	
Number of males	77,	or 64.0 %
Number of females	42,	or 35.0 %
Number of hermaphrodites	1,	or 0.83%

All individuals exclusive of adults

Total number sexed	993	
Number of males	497,	or 50.05%
Number of females	482,	or 48.53%
Number of hermaphrodites	14,	or 1.41%

All individuals

Total number sexed	1113	
Number of males	574,	or 51.57%
Number of females	524,	or 47.08%
Number of hermaphrodites	15,	or 1.35%

The variation in the percentage of occurrence of hermaphrodites in the different developmental stages has little significance because of the large probable error due to the small number of specimens examined. It should be noted that the nearest approximation to the general average, 1.35 per cent., occurs in the case of those stages in which there were the largest numbers examined. The extremely low percentage in the adult group is noteworthy and, though probably due to the small number of specimens used, may conceivably indicate a lower degree of viability in the case of hermaphrodites in adult life. The discrepancy in percentage of males and females in the adult group may also indicate a difference in viability, but is more likely to be due to the fact that the collections used were made mainly in the spring when the females would be more difficult to find because during the egg laying period they are under large rocks in the deeper water.

The fact of real significance is that of the existence of hermaphroditism in every developmental stage, since together with the approximate equality of the two sexes, it serves to eliminate any claim that the condition in question is, in this species, merely a transitory one.

This establishment of a fairly constant percentage of occurrence of hermaphroditism in *Eurycea bislineata* suggests the possibility that a search for the phenomenon in other urodeles might reveal a like frequency of occurrence. The urodeles offer an inviting field for such investigation since so little has been done with them in connection with the problem in contrast to the large amount of attention which has been given to the anurans.

Description of Representative Cases of Hermaphroditism in *Eurycea bislineata*.

Adult Stage.

The one hermaphroditic adult found was an individual of 65 mm. in length which had the external characteristics of a female,

as shown by the presence of a spermatheca in the dorsal wall of the cloaca, and the shape of the head. The appearance of the gonads *in situ* is shown in Fig. 1 *B*. For comparison, a typical ovary and a typical testis from animals of approximately the same size and collected at the same time (July 3, 1915) are shown in Figs. 1 *A* and 1 *C*. The smaller size of both testicular and ovarian parts of the ovo-testis as compared with the typical male and female gonad respectively will be noted. Moreover, while the reproductive ducts (Wolffian and Müllerian respectively) of the normal male and female have approximately the form characteristic of

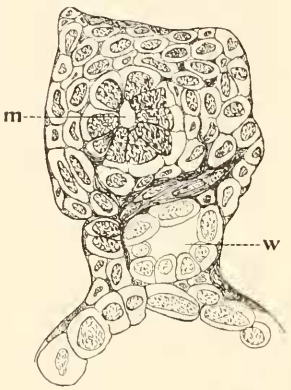

FIG. 3. Cross section showing (*m*) the Müllerian and (*w*) the Wolffian ducts in the adult hermaphrodite (cf. Fig. 1 *B*) (× 400).

the mature condition, the hermaphrodite showed macroscopically apparently a single slender straight duct upon each side following the lateral border of the mesonephros. Subsequent microscopic examinations of cross sections of this, however, revealed the presence of two ducts (cf. Fig. 3) the more lateral of which, the Müllerian, alone persists anterior to the mesonephros. This sexually indifferent condition of the ducts is identical with that shown by cross sections, made previous to this investigation, through the body of a 66 mm. immature adult male, the testes of which also correspond histologically almost exactly to the testicular portions of the hermaphroditic gonad and were thus used as a typical male control in the microscopic study.

The fat bodies in the hermaphrodite were especially large, and when the body cavity was first opened completely obscured every-

thing beneath them, making their removal necessary for the study of the gonads. The right gonad is the larger and in a macroscopic examination seems to be primarily a testis with characteristic pigmentation and conspicuous lobules. The pigmentation is, however, somewhat lighter in color than that usually found in the adult testis. The length of the testicular portion of the right gonad is 4 mm., while that of the testis shown in Fig. 1 *A* is 5.25 mm. Posterior to this testicular portion, occurs a more slender unpigmented structure in which ten large unmistakable ova, together with smaller ones, may be seen. Its general resemblance to an ovary is seen by comparison with the ovaries of the 60 mm. adult female shown in Fig. 1 *C*.

The left gonad is longer and more slender as a whole than the right. This is due to the greater length of the ovarian part, the testicular region being smaller than that of the right gonad (2.75 mm. as compared with 4 mm.). Moreover the testicular pigmentation is confined to the anterior region of the gonad and is still lighter in color than that of the right gonad. The characteristic lobules are present, but there is less differentiation of the testicular from the ovarian region, the two seeming to grade into each other insensibly. In this gonad 14 large ova are in evidence as well as numerous smaller ones. At the extreme posterior end of the left gonad there is a small semi-detached ovarian lobe.

The hermaphrodite had not been preserved originally for histological study, since the animal had been killed in 5 per cent. formalin and had been kept in this fluid since 1915. Nevertheless the gonads were sectioned, and, in spite of the excessive shrinking which is especially evident in the separation of the cysts which make up the testicular lobules, the characteristic structure of both the male and the female components was shown with unmistakable clearness.

Figure 4 shows a cross section through a region where, in a macroscopic examination, the right gonad had the appearance of a testis and the left one the appearance of an ovary. In general this section shows the typical testicular structure of the right gonad, with lobules, each made up of a number of component cysts of male cells, arranged radially about a central collecting duct. A single large ovum appears in the section, however, completely fill-

ing one of the lobules and thus apparently the equivalent of many cysts. The characteristic anabolic and katabolic nature of the female and male cells respectively is thus well exemplified. The female cell grows, the male cell divides. In the whole series of sec-

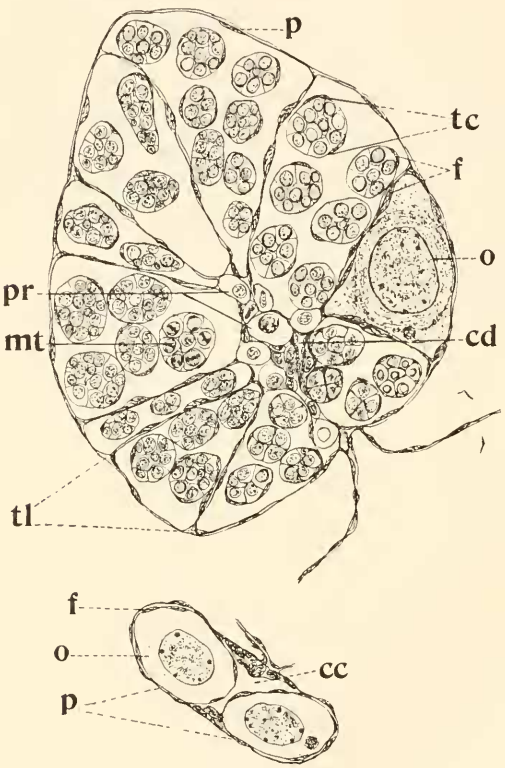

FIG. 4. Cross section through the gonads of the adult hermaphrodite at the level indicated in Fig. 1 *B* by the line 1–2 (× 87). The two gonads are brought nearer together in the drawing than their actual position. *Cc*, central cavity of ovarian region; *cd*, collecting duct of testicular region; *f*, follicles of both ova and testicular cysts; *mt*, spermatogonial cells in mitosis; *o*, ova (primary oöcytes); *p*, peritoneal investment; *pr*, primordial germ cells; *tc*, testicular cyst; *tl*, testicular lobule.

tions through the gonads no fewer than ten such ova were found in the testicular portion of the right gonad and six in that of the left. All were, like the one shown in Fig. 4, in an apparently normal state of development, manifesting no incipient signs of degeneration such as Crew ('21) reports to be the case in Anura whenever

female elements are found in parts which are primarily male in character. In fact no difference could be detected between the ova among the testicular lobules and those of the more distinctly ovarian part of the gonads except that the former had advanced further in the matter of accumulation of layers of yolk.

There was much mitotic activity in progress in the testicular lobules, the same stage of mitosis being exhibited by all the cells of a given cyst, a condition which is to be expected if one postulates their formation by repeated divisions of a single primordial spermatogonium. Thus the male elements, like the female, have every appearance of undergoing perfectly normal development. In the transition region from the testicular to the ovarian part of the gonad, small testicular lobules appear which are somewhat degenerate in character.

The posterior part of each gonad shows the typical ovarian structure as demonstrated by the section of the left gonad in Fig. 4, with large central cavity surrounded by ova, each within its layer of follicle cells.

The microscopic condition thus shown seems scarcely more advanced than that pictured and described by Chapin ('15) in the gonads of her 46 mm. hermaphroditic "larva," which, in the absence of the more exact criteria of developmental stages of the whole larval life such as we are here making use of, was designated as a "larva" in the sense that it had not yet undergone transformation. In reality it was probably an individual which was approaching metamorphosis if not in actual metamorphic condition. In general this species shows much normal variation in the developmental condition of the gonads at transformation and it is thus not surprising that one individual previous to transformation should be in the same condition as another which has already transformed.

Our adult hermaphrodite is noteworthy, not only because it shows that the condition is not merely a juvenile one, but also because so far as external characters are concerned it appears to be a female. These characters, it must be confessed, are not of a very decided nature in this species, the presence of a spermatheca being, indeed, the only unquestionable one. Moreover, the cloacal papillæ which are the characteristic male structures, might

not have appeared in so immature an individual and thus one cannot be sure that later the cloaca might not have shown male as well as female structures.

Crew ('21) in his summary of the recorded cases of abnormality of the reproductive system, says that of the 30 frogs of which sufficient details were given as to their secondary sexual characters, 25 (83.3 per cent.) were definitely and typically males; 4 others were definitely but imperfectly male (13.3 per cent.); and in the remaining case, a *Rana temporaria* described by Huxley ('20), the secondary sexual characters were female (3.3 per cent.). He says: "The abnormalities which have been recorded can be so tabulated that the first case most nearly approximates to the normal female and the last the typical male, with respect to the nature of both primary and secondary sexual characters. Thus arranged it is seen that the cases furnish an almost complete series of gradations which range from an individual almost completely female, to one almost completely male, and that the conditions found readily appear to be merely graded stages of a single process."

All of our other hermaphroditic examples of *Eurycea* were in too early a stage of development for secondary sexual characters to have appeared. However, so far as the condition of the gonads alone was concerned the same sort of graded series was found as that described by Crew in the frogs.

More thorough microscopic examination of gonads might, by disclosing occasional ova among the lobules of an otherwise normal testis or a few testicular elements concealed by the large ova of an ovary, yield a more complete seriation. At least the conditions shown by *Eurycea* indicate that in this species the hermaphroditic condition cannot be interpreted as always a modification of the same sex.

Metamorphic Stage.

In the more advanced developmental stages, as in the case of the adults, the sexing of the specimens consisted in distinguishing between a large unpigmented ovary full of bulging ova and a more slender, heavily pigmented testis with, of course, attention directed toward the detection of any combination of the two, which would mean an hermaphroditic condition. Fig. 5 *B* shows the hermaph-

roditic gonads of a 42.4 mm. animal in the advanced metamorphic stage. The gonads are essentially male so far as general shape and slight characteristic pigmentation are concerned, and there are visible in them ten unmistakable ova. Figs. 5 *A* and 5 *C* represent the gonads of a typical male and female collected on the

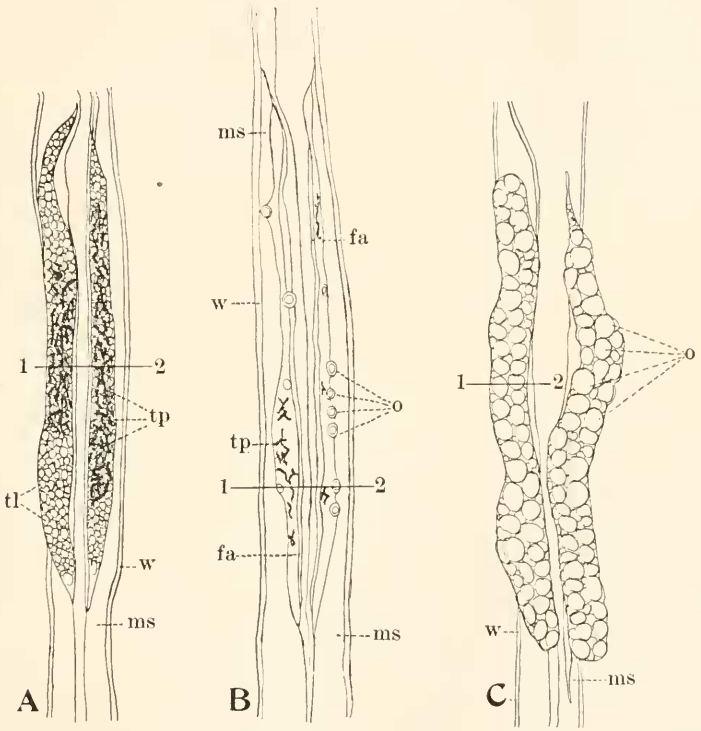

FIG. 5. Camera lucida drawings of the ventral view of gonads of (*A*) an incipient metamorphic male, length 40.9 mm.; (*B*) an advanced metamorphic hermaphrodite, length 42.4 mm.; and (*C*) a premetamorphic female, length 40.2 mm. (× 15). *Fa*, fat bodies; *ms*, mesonephros; *o*, ova; *tl*, testicular lobules; *tp*, testicular pigment; *w*, Wolffian duct. The levels of the sections of the gonads shown in Fig. 6 are indicated by the lines 1–2.

same date and of approximately the same size and stage of development, which may be used for comparison. As noted before, the smaller size of the hermaphroditic gonad, and of the ova present in it, is obvious.

Cross sections 10 micra thick were made through all three pairs of gonads and were stained, some with Delafield hæmatoxylin,

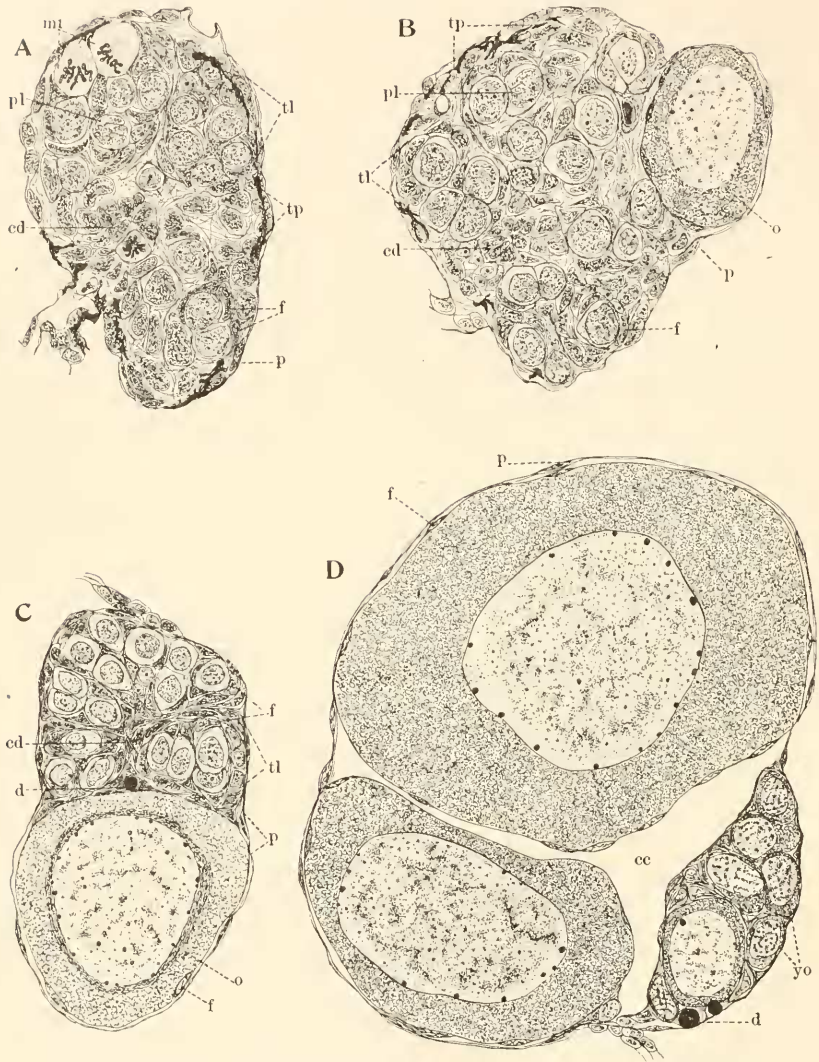

FIG. 6. Cross sections, at the levels indicated in Figure 5 by the lines 1–2, showing (*A*) the right gonad of an incipient metamorphic male; (*B*) the right gonad of an advanced metamorphic hermaphrodite; (*C*) the left gonad of the same individual; and (*D*) the right gonad of a premetamorphic female (× 365). *Cc*, central cavity of ovary; *cd*, collecting duct of the testis and testicular region of the hermaphroditic gonad; *d*, degenerating cells; *f*, follicles; *mt*, spermatogonial mitoses; *o*, ova (primary oöcytes); *p*, peritoneal investment; *pl*, polymorphonuclear germ cells (primary spermatogonia); *tl*, testicular lobule; *tp*, testicular pigmentation; *yo*, young primary oöcytes.

others with iron hæmatoxylin, and still others with safranin and light green. The lobules of the normal testis (Fig. 6 *A*) are made up of spermatogonia surrounded singly or in small groups with follicle cells, with which the cysts later to be formed by the division of these spermatogonia will be covered. Typical spermatogonial mitoses are seen in this and in other sections. In the hermaphroditic gonads (Figs. 6 *B* and *C*) we find a testicular structure corresponding in general to that shown by the normal testis, with typical mitoses in evidence. At the level shown in *C* in which the ovum constitutes practically half of the total diameter of the gonad, the testicular part is not quite so far advanced as in *B*, but is, in fact, in much the same condition as the more anterior region of the normal testis. The ova shown in both of these sections are typical, as will be seen by comparison with the section of the normal ovary (Fig. 6 *D*), although they are not equal in size to the larger ones of the normal ovary.

Incipient Premetamorphic Stage.

In the examination of younger stages in which little or no testicular pigment had developed, reliance for the diagnosis of the sex had to be based upon the shape of the ovary with its protruding ova to distinguish that organ from the slender testis or from the testis with female elements present in it.

Figure 7 *B* shows the general appearance of the hermaphroditic gonad of a 36 mm. incipient premetamorphic individual, and Figs. 7 *A* and *C* show gonads of a typical male and female of about the same size and developmental condition. The smaller size of the hermaphroditic gonad is again evidenced. The ova are of about the size of the smallest seen in the normal ovary.

The anterior part of the reproductive organs in each case was sectioned transversely for the purpose of studying the relation of the ducts; while the posterior part, including, in fact, the major part of the gonads themselves, was sectioned horizontally. Delafield hæmatoxylin and iron hæmatoxylin staining were used.

Histologically the developmental condition of these gonads as shown in Fig. 8 *A, B,* and *C* is not essentially different from that of the metamorphic stage, except that both in the normal testis and in the testicular region of the hermaphroditic gonad there are

more single spermatogonia and fewer in groups, although the lobulated structure of the gonad is evident. There were fewer instances of spermatogonial mitosis, none, in fact, discovered in the hermaphrodite, the sections of which, however, were somewhat

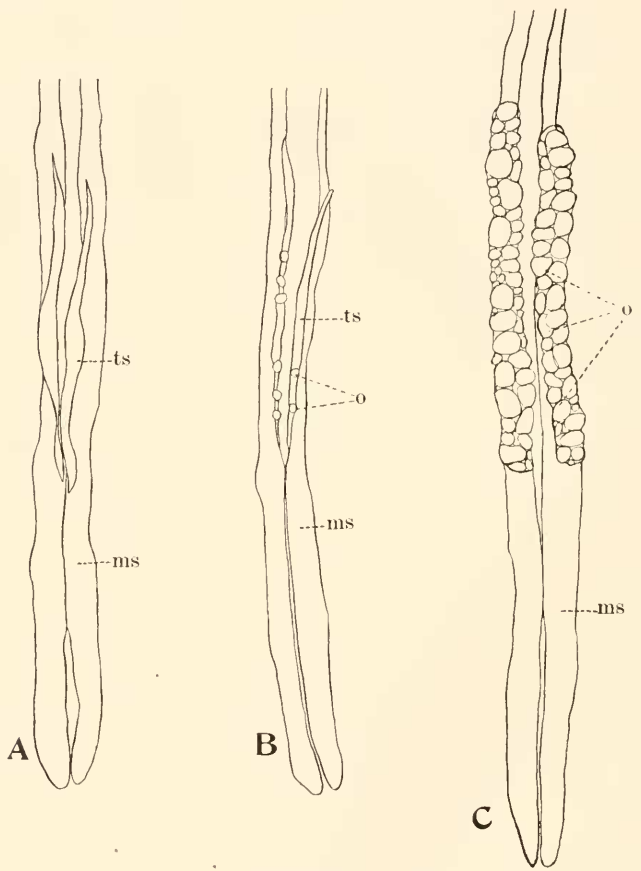

FIG. 7. Camera lucida drawings of the ventral view of the gonads of (*A*) an incipient premetamorphic male, length 37.4 mm.; (*B*) an incipient premetamorphic hermaphrodite, length 36 mm.; and (*C*) an incipient premetamorphic female, length 36.2 mm. (× 15). *Ms*, mesonephros. *o*, ova; *ts*, testis and testicular portion of hermaphroditic gonad.

fragmentary. In every other particular of cell arangement and nuclear structure the testicular regions of the hermaphrodite were identical with the normal testis. The ova of the hermaphrodite,

though of smaller size, were perfectly normal in appearance. They have the typical relationship to the testicular lobules, and, owing to their earlier stage of growth, do not bulge out so conspicuously from the surface of the gonad as in the case of the metamorphic stage (cf. Fig. 6 *B* and *C*).

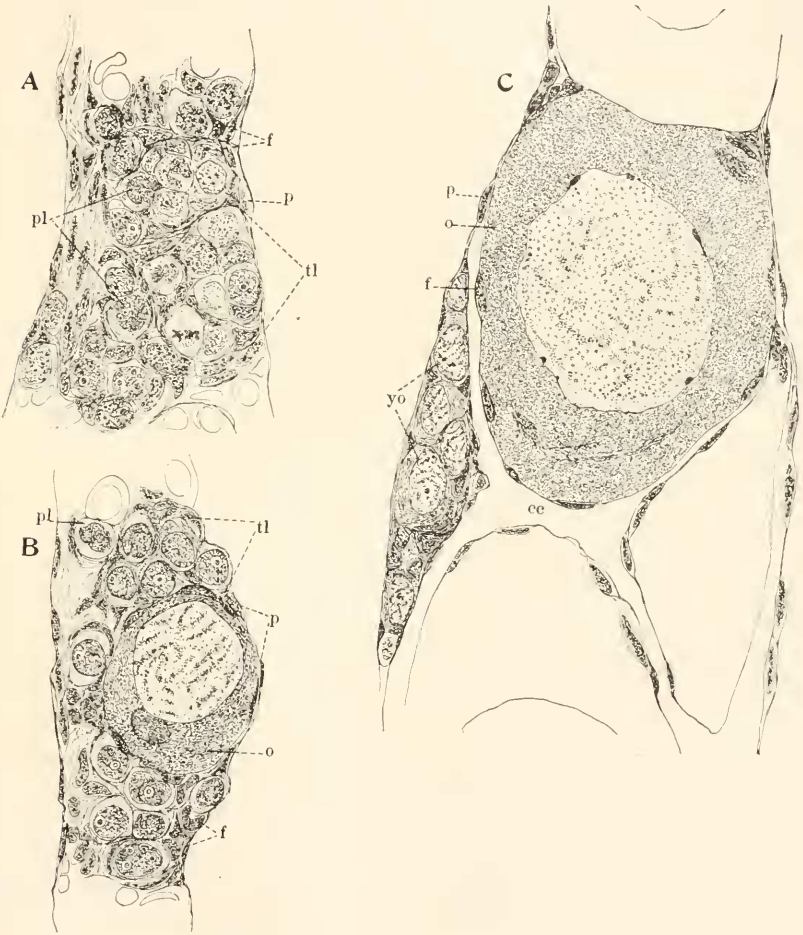

FIG. 8. Horizontal sections through corresponding regions of the gonads shown in Fig. 7 of (*A*) male; (*B*) hermaphrodite; (*C*) female (× 356). *Cc*, central cavity; *f*, follicles; *o*, ova (primary oöcytes); *p*, peritoneal investment; *pl*, polymorphonuclear germ cells (primary spermatogonia); *tl*, testicular lobule; *yo*, young primary oöcytes.

Typical Larval Stage.

With the younger stages, macroscopic evidence could be relied upon still less for sexing, although normal ovaries are easily recognizable if the growth period of the ova has been well entered upon, and such normal female gonads packed with growing ova of approximately uniform size have been distinguished macroscopically in larvæ as small as 25 mm. in length, though our percentage data (p. 8) did not include individuals under 27 mm. in length. The difficulty in sexing lies in the uncertainty as to the presence of male elements in gonads in which the ova are few in number but unmistakable. We have not as yet examined microscopically large numbers of gonads of young larvæ. However, in looking over our laboratory sets of serial sections of larvæ collected in September or early October, ranging in length from 17 to 25 mm. and presumably about 12 weeks old, we find that while a few of them show a condition which might be considered as sexually indifferent in that the gonads are made up of typical primordial germ cells, each with its investment of follicle cells, arranged in single rank about a central cord, a larger number of those examined, including some of the smallest individuals, show practically all of the germ cells in early maturation stages (leptonene and pachytene) or as growing oöcytes. Such an individual seems to us to be a female, since other individuals show gonads made up of more numerous, smaller germ cells grouped in such a manner as to suggest at once the incipient lobules of a typical testis. In such gonads the germ cells show no maturation phenomena, although mitosis is ocasionally seen. For the most part the nuclei are either polymorphic, or in rounded form with one or more conspicuous nucleoli. As we have very few data as to the condition of the gonads the following spring, we can only express here our tentative opinion that this species, in spite of its prolonged larval life, exhibits no such early larval maturation of male germ cells synchronously with that of the female germ cells, as has been described by Swingle ('21) for *Rana catesbeiana.*

To push our power of diagnosis of sex and recognition of possible hermaphrodites back into these early stages demands as a basis, not only a careful and thorough cytological investigation of the origin and differentiation of the germ cells such as that of

Bouin ('01) and Dustin ('07) for other species of Amphibia (mainly *Anura*), and Okkelberg ('21) for the brook lamprey, but also a complete bridging over of the gap between the early developmental phenomena and the seasonal sexual phenomena of adult life.

With regard to the bearing which occasional hermaphroditism such as this has upon its regular occurrence in certain species of animals, and upon the significance of the phenomenon in general, two opposing views are held. One of these, as set forth by Doncaster ('14), regards hermaphroditism not as a primitive but as a purely secondary condition. This opinion is based mainly on the fact that the hermaphroditic species of animals are, for the most part, highly specialized ones. Sporadic hermaphroditism is thus considered an example of variation along this same direction.

The other view is that which has recently found so vigorous a supporter in Jordan ('22), that hermaphroditism, at least in the vertebrate group, is a primitive character. Jordan points out "the abundant evidence of a normal hermaphroditic condition either adult or juvenile, among lower vertebrates (*e.g.*, tunicates, cyclostomes, probably some Amphibia)," and that "the early gonads with their primordial germ cells appear identical." This view of the primitive character of hermaphroditism naturally goes hand in hand with the theory that sex determination is a matter of differential metabolism and that forms in which sex determination has become bound up in the chromosomes represent a higher stage in metabolic control of the developing organism.

Jordan points out the peculiar interest presented by the case of amphibians in this connection, since most investigators have failed to find any evidence of a sex chromosome in this group, although King ('12) describes it for *Necturus maculatus*, Levy ('15) for *Rana esculenta*, and Swingle ('17) for *Rana pipiens*. In a later paper, however, Swingle ('21) questions the correctness of his own earlier identification of an accessory chromosome in *Rana pipiens* and suggests the strong probability that Levy may also have been mistaken.

Jordan makes the suggestion that the Amphibia may constitute a group in which the evolution of the sex chromosome as a separate element can be traced, and in which also a general ten-

dency toward juvenile hermaphroditism bridges the gap between lower vertebrates where functional hermaphroditism occurs in certain classes and higher vertebrates where the condition occurs only as an anomaly. Swingle has done much to dispel the idea of juvenile hermaphroditism in the anurans. Cases of hermaphroditism such as we have here described in *Eurycea bislineata* give every evidence of being a permanent rather than juvenile condition. On the other hand, although we have thus far found no evidence that these permanent hermaphrodites arise out of an earlier condition in which the gonads have the potentiality for both sexes and may thus be regarded as capable of producing either males, females, or hermaphrodites, we do not feel that our investigation of these early stages has been sufficiently extensive to warrant us in excluding this possibility. In the absence of evidence of a chromosomal control of sex determination in this species, and indeed in Amphibia in general, one should maintain an open mind toward other possibilities. Much further investigation of the subject is therefore obviously needed and is now in progress.

Summary.

1. True hermaphroditism occurs in approximately constant proportions in every developmental stage of *Eurycea bislineata* from typical larval to adult.

2. The percentage of occurrence of hermaphrodites in this species, based upon the examination of 1,113 individuals, is 1.35 per cent.

3. There are now on record 18 cases of hermaphroditism in urodeles. The first is that of La Valette St. George ('95) in *Triton tæniatus*, the second is that discovered by Chapin ('15) in *Eurycea bislineata*, the third is that of Křizenecky ('17) in *Triton cristatus*, and the other 15 cases, in *Eurycea bislineata*, are presented in this paper.

Department of Zoology, Smith College,
Northampton, Mass.
November 13, 1923.

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